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EXPERIMENTS ON THE RÔLE OF LYMPHOID TISSUE IN THE RESISTANCE TO EXPERIMENTAL TUBERCULOSIS IN MICE.

II. Effect of Cancer Immunity on Resistance to Tuberculosis.

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(Received for publication, January 20, 1917.)

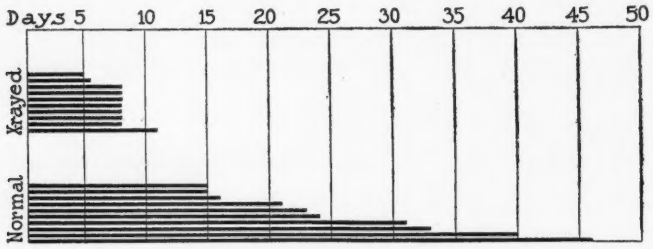
An investigation recently reported from this laboratory seems to bear out the conception that the lymphocyte plays a part in the resistance of the animal to tuberculous infection.[1] This work had for its starting-point the observation of Lewis and Margot[2] that mice experimentally infected with bovine tubercle bacilli developed splenic enlargement. Lewis and Margot also showed that animals splenectomized about 3 weeks before the injection of the tubercle bacilli exhibited greater resistance to the infection than did intact animals. In a study in this laboratory of blood changes after splenectomy, it was observed that the majority of mice so treated developed a marked lymphocytosis by the 19th to the 21st day after the operation.[3] It was thought probable, therefore, that this lymphocytosis might be a factor in causing the greater resistance displayed by the splenectomized animals. The following experiments confirm this view. Mice splenectomized and then exposed to repeated small doses of x-ray, which had been demonstrated to affect primarily the lymphoid

[1] Murphy, Jas. B., and Ellis, A. W. M., *J. Exp. Med.*, 1914, xx, 397.

[2] Lewis, P. A., and Margot, A. G., *J. Exp. Med.*, 1914, xix, 187.

[3] L. B. Lange and Jas. B. Murphy, in an unpublished study of the blood of fifty mice before, and at intervals after splenectomy, showed that immediately after the operation there was a fall in the total lymphoid count. But by the 19th to the 21st day, 75 per cent of the animals counted showed an average increase of 11,000 lymphocytes per c.mm., or a gain of over 100 per cent above their normal count. The 25 per cent which did not gain averaged a loss of about 8 per cent of their former count.

organs,[4] proved to be more susceptible instead of more resistant to infection than were either normal animals or animals splenectomized alone (Text-fig. 1). Intact x-rayed mice were likewise highly susceptible to infection with the bovine tubercle bacilli. Morton[5] has observed also that the x-rayed guinea pig is more susceptible to infection with the human type of the tubercle bacillus than is the normal animal.



TEXT-FIG. 1. Each horizontal line represents the time of survival of a mouse after infection with bovine tubercle bacilli. The first group received a series of small doses of x-ray before inoculation, and the average duration of life was 7.8 days. The second group was normal and averaged 26.4 days of life after inoculation.

The Use of Cancer Immunity as a Method for Producing Lymphocytosis.

As this evidence is of an indirect nature, it seemed desirable to obtain more direct data bearing on this conception. The present communication deals with the influence of a pronounced lymphocytosis induced by other means than splenectomy on the resistance of the mouse to tuberculous infection. We have already shown that mice immunized against and then inoculated with one of the transplantable mouse cancers developed a marked lymphoid reaction in the blood which lasted several weeks.[6] This method, therefore, affords a convenient way of obtaining what may be regarded as non-specific high blood lymphocytic reaction which lasts long enough to answer the experimental requirement.

[4] Murphy, Jas. B., *J. Am. Med. Assn.*, 1914, lxii, 1459.
[5] Morton, J. J., *J. Exp. Med.*, 1916, xxiv, 419.
[6] Murphy, Jas. B., and Morton, J. J., *J. Exp. Med.*, 1915, xxii, 204.

Experiment 1.—Twenty mice were injected subcutaneously with 0.3 cc. of defibrinated mouse blood. These, with twenty control animals, were inoculated 10 days later with a transplantable mouse carcinoma. At the end of 3 weeks 85 per cent of the control mice had developed tumors, while only one of the surviving sixteen immunized animals showed tumor. The fifteen immune animals, with ten normal mice from the same lot were each injected intraperitoneally with 3 mg. of a 9 day culture of bovine tubercle bacilli from a glycerol agar slant, taken up in 0.5 cc. of normal salt solution. The mice were placed in individual jars in order to prevent loss from epidemic disease. Autopsies were performed as soon after death as possible, and films were taken from the peritoneal fluid, kidneys, liver, spleen, lungs, and heart's blood. The gross and micropathology will not be described at this time. The distribution of the microorganisms is shown in Table I. The average survival after inoculation for the control animals was 20.3 days, while that of the cancer immune mice was 47.7 days; that is, they lived more than twice as long. The rate at which the mice died is shown in Text-fig. 2.

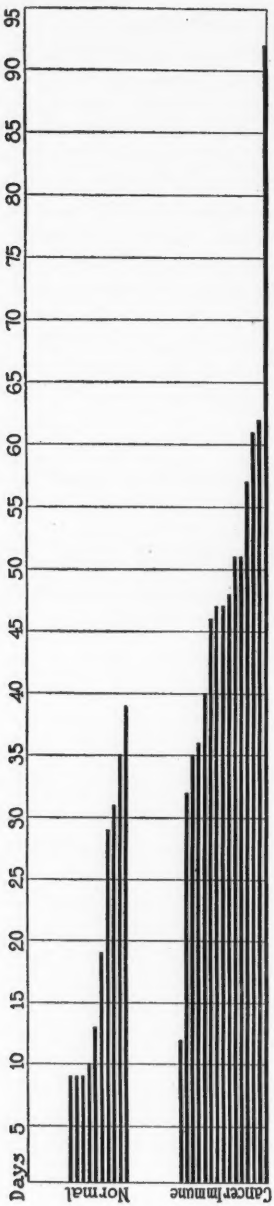
TABLE I.

Distribution of Tubercle Bacilli in the Cancer Immune Mice and the Control Mice from Experiment 1.

Group.	Average time of survival.	Percentage of animals with tubercle bacilli in various organs.					
		Exudate.	Spleen.	Liver.	Heart's blood.	Lungs.	Kidneys.
	days	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Controls.....	20.3	100	80	70	50	90	60
Cancer immune mice	47.7	86.6	100	73.3	66.6	86.6	60

Experiment 2.—Nineteen mice were injected with an immunizing dose of 0.3 cc. of defibrinated mouse blood. 10 days later these animals, with ten controls, were inoculated with a transplantable carcinoma. After 3 weeks, four of the nineteen immunized animals and nine of the controls had developed tumors. At this time all the immunized animals, including the four which had developed tumors, with ten normal mice as controls, were each inoculated intraperitoneally with 3 mg. of a 27 day old culture of bovine tubercle bacilli[7] taken up in 0.5 cc. of normal salt solution. The mice were kept throughout the experiment in individual jars. The distribution of the organisms at autopsy is shown in Table II. The average duration of life after inoculation for the control animals was 14.7 days, and for the cancer immune animals 24.3 days. The four animals which had been immunized but developed tumors lived on an average of 17.7 days. The rate at which the mice died is shown in Text-fig. 3.

[7] The same culture was used in this experiment as in the first, but in the interval it had been passed through an x-rayed guinea pig.

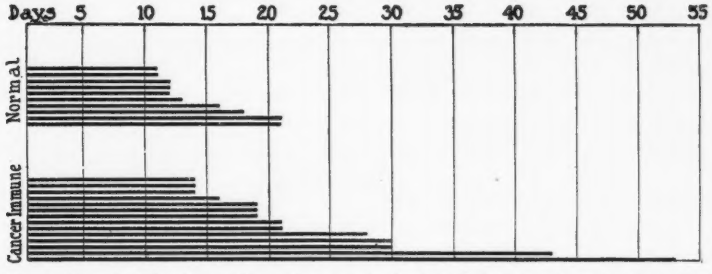


TEXT-FIG. 2. The horizontal lines show duration of life after inoculation of normal and cancer immune mice with tubercle bacilli. The average time of survival of the normal animals was 20.3 days, and of the cancer immune animals 47.7 days.

TABLE II.

Distribution of Tubercle Bacilli in the Cancer Immune Mice and the Control Mice from Experiment 2.

Group.	Average time of survival.	Percentage of animals with tubercle bacilli in various organs.					
		Exudate.	Spleen.	Liver.	Heart's blood.	Lungs.	Kidneys.
	days	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Controls.....	14.7	100	100	81.9	0.9	100	100
Cancer immune mice.....	24.3	100	100	73.3	13.3	80	73.3



TEXT-FIG. 3. The lines show the survival of individual mice after inoculation with tubercle bacilli. The normal animals averaged 14.7 days, while the cancer immune animals averaged 24.3 days.

The two experiments agree in showing that mice first immunized to, and then inoculated with a transplantable mouse cancer exhibit a greater resistance to bovine tuberculosis than do normal animals. On the other hand, we have noticed that while animals ineffectively immunized against cancer show a definite lymphoid reaction in the blood, it is less marked than when the immunity is perfect. In this connection, it is of interest to observe that the four mice of this kind in Experiment 2 outlived the controls but succumbed earlier than the completely immune ones. However, a larger series of animals would be needed to establish this point.

Destruction of Lymphocytes in Cancer Immune Mice and Resistance to Tuberculous Infection.

If the lymphocytic reaction in the cancer immune animals is the factor which determines the enhanced resistance to tuberculous

infection, the destruction of these cells by means of the x-ray should reduce resistance to a point even below that of normal animals. The following experiment was performed with this in view.

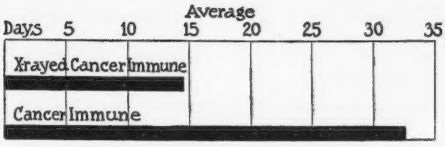
Experiment 3.—Thirty-eight mice were immunized with 0.3 cc. of defibrinated mouse blood, and 10 days later, with ten control animals, were inoculated with a transplantable mouse carcinoma. Five of the thirty-eight immunized animals developed cancers, as did all the controls. 2 weeks after the cancer inoculation, eighteen of the immune animals were given a daily dose of x-ray until they had received seven exposures. At this time, or 3 weeks after the cancer inoculation, both the x-rayed and non-x-rayed cancer immune mice were each inoculated intraperitoneally with 2 mg. of a culture of bovine tubercle bacilli. The same observations were made as in the two previous experiments. The distribution of the microorganisms is shown in Table III. The average duration of life after the tubercle bacilli inoculation in the x-rayed cancer immune animals was 14.5 days and in the non-x-rayed cancer immune animals 32.5 days (Text-fig. 4).

TABLE III.

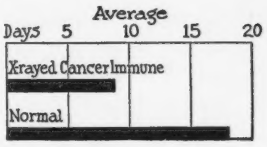
Distribution of Tubercle Bacilli in the Organs of Animals from Experiment 3.

Group.	Average time of survival.	Percentage of animals with tubercle bacilli in various organs.					
		Exudate.	Spleen.	Liver.	Heart's blood.	Lungs.	Kidneys.
	days	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
X-rayed cancer immune mice	14.5	100	94.2	77.7	5.5	33.3	55.5
Intact cancer immune mice	32.5	95.2	80.9	66.6	4.7	28.5	33.3

Experiment 4.—Ten mice, which had been proved immune to cancer as a result of an injection of defibrinated blood, were given a series of doses of x-ray sufficient to reduce their lymphoid tissue. 3 weeks after the cancer inoculation, and 24 hours after the last x-ray exposure, the ten mice, with ten normals, were injected intraperitoneally with 3 mg. of a 3 weeks old culture of bovine tubercle bacilli taken up in 0.5 cc. of normal salt solution. All the animals at this time were in excellent physical condition. The same general routine was observed as in the former experiments. The x-rayed cancer immune animals died off very rapidly, the average duration of life being 8.9 days. The control mice died at a much slower rate and averaged 18 days of life after the inoculation; that is, they lived twice as long as the x-rayed series (Text-fig. 5).



TEXT-FIG. 4.



TEXT-FIG. 5.

TEXT-FIG. 4. The average duration of life of cancer immune mice subjected to repeated small doses of x-ray before inoculation with tubercle bacilli compared with the duration of life of intact cancer immune animals inoculated with the same infecting agent.

TEXT-FIG. 5. The average duration of life of cancer immune animals subjected to repeated small doses of x-ray before inoculation with tubercle bacilli compared with the duration of life of normal mice inoculated with the same infecting agent.

These experiments demonstrate conclusively that the enhanced resistance of cancer immune mice to tuberculous infection can be set aside or even reduced to a state of increased susceptibility by destroying the lymphocytes by means of exposure to x-rays.

DISCUSSION.

The experiments reported were not undertaken with the idea of establishing a relationship between cancer and tuberculosis, as we know of no sufficient reason to assume the existence of such a relationship. However, it has long been believed that some such specific antagonism between the two conditions exists. But considering that the ages at which cancer and tuberculosis reach their highest incidence are widely divergent, and that cancer rarely, if ever, attacks a debilitated individual, this idea of a specific antagonism would seem to have little basis in fact. That the two diseases may occur simultaneously in the same individual is borne out by many reports made in recent years.

It is probable that the lymphoid tissue of the body may be a determining factor in the phenomena of resistance in both tuberculosis and cancer, and yet the *modus operandi* involved may be absolutely different. Indications that the lymphoid cell plays a part in resistance to tuberculosis are numerous. The constant association of

these cells with the lesions of the disease is so well recognized that it need not be gone into here. In most of the clinical blood studies of tuberculous individuals, the white cells have been analyzed from the point of view of the polynuclear cell, but as these do not increase markedly, little attention has been paid to the other white cells. However, the blood counts show an interesting variation in the lymphoid cells which has not attracted the attention it merits. The total number of lymphocytes per c.mm. of blood in a normal adult varies between 1,500 and 2,500 cells. In one case of advanced pulmonary tuberculosis reported by Emerson,[8] there were only 530 lymphocytes, and in a case of miliary tuberculosis studied by the same author, the total number was only 227. Warthin[9] reports a case of miliary tuberculosis in which the lymphocytes made up only 5.5 per cent of a subnormal white cell count. On the other hand, in cases which give a good prognosis, these cells may form 40 to 50 per cent of a white cell count somewhat higher than normal, which means a substantial increase in the lymphoid cells. Wack[10] has observed that cases of tuberculosis advancing rapidly show a decrease, and those healing or healed a corresponding increase in the mononuclear cells. While the reports in the literature are not as convincing as might be desired, yet they present indications to the effect that the lymphocytes vary directly as the degree of resistance to the tuberculous infection. To bring together the evidence which bears on the participation of the lymphocyte in resistance to tuberculous infections, we have (*a*) its constant association with the pathologic lesions of the disease, (*b*) its fluctuation in the blood with the progress of the infective process, (*c*) the reduced resistance to the infection in animals depleted of their lymphoid tissue by means of x-ray, and (*d*) a marked enhancement of resistance to the infection displayed by animals with a high lymphocyte count resulting from the establishment of cancer immunity.

[8] Emerson, C. P., Clinical Diagnosis, Philadelphia and London, 4th edition, 1913, 624.

[9] Warthin, A. S., *Med. News*, 1896, lxviii, 89.

[10] Wack, P., *Deutsch. Arch. klin. Med.*, 1914, cxv, 596.

SUMMARY.

Mice so x-rayed as greatly to reduce the lymphoid tissue are rendered highly susceptible to tuberculous infection. On the other hand, when a marked lymphocytosis is induced by first immunizing mice against, and then inoculating them with cancer, the resistance to tuberculous infection is greatly enhanced. This heightened resistance may be set aside and even changed to a state of increased susceptibility to the infection by again depleting the lymphocytes by means of the x-ray.

THE TRANSPLANTATION OF SPLENIC TISSUE INTO THE SUBCUTANEOUS FASCIA OF THE ABDOMEN IN RABBITS.

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(*From the H. K. Cushing Laboratory of Experimental Medicine of Western Reserve University, Cleveland.*)

Plate 54.

(Received for publication, January 3, 1917.)

The reactions of certain structures of the spleen, for example the pulp cells and the endothelium of the sinuses, to many varieties of toxins have been extensively studied. On the other hand, little is known of the nature and function of the Malpighian bodies. While morphologically they resemble lymphoid tissue, the fact that this tissue rarely shows the reactions seen in other lymphoid tissues to bacterial toxins like typhoid, streptococcus, etc., may be of significance. In certain states, however, as in lymphatism, exophthalmic goiter, etc., this tissue, like the thymus, shares in the general lymphoid hyperplasia. Then too, its arrangement as an envelope about the arteries is not duplicated elsewhere in the body. The question of the relative importance of the structures in regeneration also has received little attention. Are the Malpighian bodies, the pulp cells, and the sinuses separate tissues with separate functions, or are they more interrelated functionally and morphologically than their anatomical appearances indicate? It occurred to us that transplantation, if this was possible, would throw some light on the subject of regeneration and possibly on the relative value of the tissues in this reaction. We have not been able to find any record of the transplantation of splenic tissue where the grafts were studied from these viewpoints or from the standpoints of the growth and permanence of the grafts.

TABLE I.

Transplantation of Spleen.

Rabbit No.	Sex.	Date of splenectomy.	Date of thymectomy.	Date of transplantation.		First examination.		Final examination.		Histological examination.
				Autotransplantation.	Homoiotransplantation.	Day.	Condition.	Day.	Condition.	
		1915	*1915*	*1915*	*1915*					
1	Male.	Aug. 26			Dec. 16	56	—	56	—	Negative (infected); infiltrated with pus cells and edematous.
2	"	" 26	Nov. 29		Nov. 29	15	—	15	—	No trace of spleen transplant found.
3	Female.	" 26	" 29		Dec. 16	65	—	65	—	Negative at examination; area not removed.
4	Male.	Nov. 5	" 29		Nov. 29	17	—	17	—	Negative; nearly absorbed; marked connective tissue reaction and invasion; persistence of the spleen trabeculæ; no lymph or pulp tissue present.
					Dec. 16	67	—	67	—	Complete absorption.
5	"	" 5	Dec. 2		" 18	63	—	63	—	Absorbed area with much yellow pigment in endothelial-like cells; some lymph tissue. This is clearly an atrophic spleen transplant with persistence of the endothelial elements.
6	Female.	" 5	" 2		" 2	14	+	14	+	Positive; marked connective tissue reaction and involution. Lymph and pulp tissues still present; highly vascular; in the process of absorption.
					" 16	65	—	65	—	Complete absorption. Several areas contain large numbers of phagocytic cells engorged with blood pigment.
7	Male.	" 5	" 2		" 16	65	—	65	—	Complete absorption.
8	"	" 5	" 3		" 16	67	—	67	—	Negative at examination; area not removed.
9	Female.	" 5	" 3		" 16	119	—	119	—	" " " " " "

10	Female.	Nov. 29	Nov. 29	Nov. 29		8	+	8	+	Positive; very well developed blood supply; marked connective tissue reaction.
11	Male.	Dec. 2	Dec. 2	Dec. 2		11	+	11	+	Positive; well organized blood supply; one large trabecula; both lymph and pulp cells present; well marked connective tissue reaction.
12	"	" 3	" 3	" 3		13	+	80	−	Negative at examination; area not removed.
13	"	" 3	" 3	" 3		13	+	13	+	Positive; in the process of absorption; marked connective tissue reaction, with ingrowth into transplant area; good blood supply.
					Dec. 16	67	−	67	−	Negative at examination; area not removed.
14	Female.	" 16	" 16	" 16		67	++++	325	++++	Transplant 4 by 2.5 by 2 mm.; whole transplant shows usual splenic vascularity; encapsulated; typical Malpighian body formation, trabeculæ, pulp, and sinus formation.
15	"	" 18	" 18	" 18		65	−	65	−	Complete absorption.

Many experiments of dislocation, often misnamed transplantation have been reported, as the experiments of Hédon[1] of pulling the organ through an abdominal wound with the blood supply intact, and suturing it into the subcutaneous tissues. Those of Lüdke,[2] of introducing bits of spleen into the spleens of alien species of animals, are not related to our problem, since heterotransplantation in mammals has never succeeded. The blood vessel suture experiments of Carrel[3] also have no bearing on the questions suggested above.

Brief mention of our first observations with spleen transplantation was made in a previous paper.[4] At that time only negative results with both auto- and homoiotransplantations had been obtained.

EXPERIMENTAL.

We now wish to report the end-results of a series of transplantations made more than a year ago. Twelve attempts at homoiotransplantation and six attempts at autotransplantation were made on fifteen rabbits. The more important data relating to each experiment are given in Table I.

Method.

The method employed is the same as that used by us in the transplantation of ductless gland tissues, and consists of transferring small sections of the spleen of about 2 mm. in their greatest dimension under strict aseptic precautions. Alcohol and bichloride of mercury were used instead of iodine for skin sterilization, because it is necessary to control the intake of iodine whenever the thyroid may be involved directly or indirectly in the experiment. After making a transverse abdominal skin incision about 2 cm. in length, the subcutaneous fascia is lifted with fine forceps, punctured with a cataract knife, the tissue introduced, and the fascial opening closed with a black silk ligature, which also serves to mark the site for subsequent examinations.

[1] Hédon, E., Transplantation sous-cutanée de la rate, *Compt. rend. Soc. biol.*, 1899, vi, 560.

[2] Lüdke, H., Ueber Milztransplantationen, *Münch. med. Woch.*, 1909, lvi, 1469.

[3] Carrel, A., Remote Results of the Reimplantation of Kidney and Spleen, *J. Exp. Med.*, 1910, xii, 146.

[4] Manley, O. T., and Marine, D., Transplantation of Ductless Glands with Reference to Permanence and Function, *J. Am. Med. Assn.*, 1916, lxvii, 260.

The spleen was completely removed at the time of transplantation in the group of autotransplants, while in all the homoiotransplants it was removed some time (from 112 to 24 days, in the majority about 40 days) before transplantation. This is probably too long a time interval to include a possible advantage to the transplant that might accrue from a splenic insufficiency, since Musser and Krumbhaar[5] and others[6] have shown that certain animals (dogs) usually begin to recover from the systemic effect of splenectomy, as indicated by the erythrocyte counts, in 3 to 4 weeks.

It has been definitely established that a physiological insufficiency markedly influences the growth of autotransplants of the ductless glands, but it does not influence to any extent the taking of the transplants. Likewise with homoiotransplants there is no evidence that an induced physiological insufficiency modifies the taking. Sex also does not influence the taking, growth, or rate of destruction of the transplanted tissue. The thyroid was removed in every instance but one at the time of, or shortly before transplantation without any influence on the taking or growth of the spleen grafts. Removal of the thyroid, ovaries, and spleen at one time, with immediate autotransplantation in one animal, was without effect.

Homoiotransplantation.

Direct and microscopic examinations were made in three cases on the 14th, 15th, and 17th days, while in the remaining ones it was delayed for 2 or more months. Only in one instance, the 14 day transplant, was there definite splenic tissue remaining, and in this only the lymphoid tissue and trabeculæ could be recognized. The one outstanding difference between the spleen and other tissue transplants is the marked early connective tissue reaction resembling

[5] Musser, J. H., Jr., and Krumbhaar, E. B., The Relation of the Spleen to Blood Destruction and Regeneration and to Hemolytic Jaundice. VI. The Blood Picture at Various Periods after Splenectomy, *J. Exp. Med.*, 1913, xviii, 487.

[6] Sollberger, H., Beiträge zur Physiologie der Drüsen. XIX. Fortgesetzte Beiträge zur Lehre von der Funktion der Milz als Organ des Eiweissstoffwechsels. Über die Kompensationsvorgänge nach Milzexstirpation, *Biochem. Z.*, 1913, lv, 13.

the granulation tissue formation seen in chronic inflammation. Infection from organisms in the spleen, however, is not probable, as only normal spleens from rabbits with no evidence of acute infection were used. It is more probable that the splenic tissue when transferred to the subcutaneous fascia is an active irritant. The studies of Carrel[7] on the effect of tissue extracts on the growth of connective tissue *in vitro* are of interest in this connection. He found that adult spleen extracts, thyroid extracts, and the Rous chicken sarcoma extract markedly accelerated the proliferation of connective tissue. Our observations on the irritant effect of spleen grafts confirm his observations. The effect is present in auto- as well as homoiografts, though perhaps the homoiografts excite a slightly greater connective tissue reaction. Carrel also found that thyroid extracts excite in the living animal an even more marked connective tissue proliferation both in healing skin and periosteum wounds. We have been unable to detect an excessive connective tissue proliferation around thyroid grafts. It would seem, as suggested by Carrel, that spleen extracts could be used to promote the granulation of wounds.

Autotransplantation.

Four transplants were examined on the 8th, 11th, and 13th days, respectively. All were positive and seemingly active. The 8th, 11th, and one 13th day transplants were removed for histological examination. In contrast with the homoiotransplants, examined after approximately the same period, all were positive with well established blood supplies and central necrosis, and the peripheral zone of pulp, lymphoid, and trabecular tissues was distinct. There was also the same connective tissue reaction seen in the homoiotransplants.

Of the three transplants examined at the 65th, 67th, and 80th days, complete absorption leaving large white scars at the sites had occurred in two, while in the third (Rabbit 14) a dark red, sharply circumscribed mass appeared, the size of a small wheat kernel. There were several large vessels entering it, and except for its darker color it could

[7] Carrel, Artificial Activation of the Growth *in Vitro* of Connective Tissue, *J. Exp. Med.*, 1913, xvii, 14.

easily have been mistaken for an autothyroid transplant. We did not remove it, and it was recovered at autopsy 325 days after transplantation, the rabbit having died of acute pneumonia and pleurisy. Grossly, the transplant measured 4 by 2.5 by 2 mm., and was dark bluish red in color. It had evidently reached its maximum growth before the first examination on the 67th day, since it was not noticeably larger on the 325th day than at the first examination. This could be interpreted as evidence that its growth had occurred in response to a physiological insufficiency during the time when other tissues were assuming the function lost through removal of the spleen.

Microscopic examination reveals typical congested splenic tissue embedded in striped muscle and mammary gland. The capsule is very thick as compared with transplants of ovary, parathyroid, thyroid, or adrenal, and several connective tissue bands extend from the capsule to the surrounding muscle fascia. Many large vessels run in the outer layer of the capsule. Small trabeculæ also extend throughout the gland parenchyma. Their numbers are the same that one finds in the normal spleen, while their size is proportional to the size of the organ. No attempt was made to demonstrate the presence or absence of smooth muscle fibers in the capsule and trabeculæ and no studies on the reticulum have been made. In some sections one can see as many as sixteen well defined Malpighian bodies with the characteristic central artery and radial capillary system. These Malpighian bodies are surrounded by typical splenic pulp with large highly congested sinuses. In places there are deposits of the yellowish brown blood pigment usually seen in the normal spleen pulp. We have, therefore, to deal with a small newly formed spleen developed in an entirely foreign field as regards its location, nerves, and blood vessel relations. It has all the characteristics of the normal spleen in as far as these have been investigated, both as to the number of its component structures, capsule, trabeculæ, Malpighian bodies, pulp, sinuses, and blood pigment, and their relation to each other. The trabeculæ are proportional to the size of the organ, while all the other structures are of normal proportions, suggesting that the trabeculæ play a purely mechanical part. The capsule is still relatively much thicker than that of the normal spleen,

and it seems probable that this is due to the enormous proliferation of the connective tissue which takes place around the graft very early (within 2 weeks), and also that this tends to return to relatively more normal proportions through absorption as the connective tissue becomes adjusted to its new neighbor. One of the striking facts, then, is that the regeneration involves all the structures in such a way that both their normal proportions and arrangements are wholly retained. This would suggest a fairly uniform vitality for all the component parts as well as a close functional interrelation. It may be recalled that in the adrenal the cortical cells readily survive, while the medullary cells invariably die, at least in our experience. When one recalls that in the thyroid only a narrow peripheral zone of three or four cells in thickness survives the transfer and that the whole interior portion undergoes necrosis, it is of much more significance that a fully regenerated organ should develope after transplantation of splenic tissue than in the case of such tissues as the thyroid or parathyroid which contain but a single specialized tissue.

The regeneration of Malpighian bodies is also of interest. Why does the lymphoid tissue not regenerate around the regenerated arteries running in the capsule and trabeculæ as well as in those of the interior of the lobule? The best studies on the taking of engrafted tissues indicate that the host supplies the new blood vessels to the transferred tissue rather than that it utilizes the possible surviving fragments in the graft. It is difficult to harmonize this with the known fact that the intralobular arteries of even a regenerated spleen develop this envelope of lymphoid-like cells. Either the vessels must be specific, which does not seem probable, or the lymphoid-like cells are specific in that they control and determine the blood vessel arrangement. If this is the case, we have proof that the Malpighian bodies represent a different type of lymphoid tissue, functionally as different from ordinary lymph node tissue as is thymic tissue.

The same questions must also be raised in connection with the development of the spleen sinuses as have been discussed in connection with the formation of the Malpighian bodies. The careful study of an appropriate series of these grafts will doubtless give valuable data, both as to the normal development and function of this peculiar

organ. The survival and growth of the pulp cells are more easily understood and the process is probably similar to that of other single type tissues.

The general features of the transplant are shown in the accompanying photomicrographs (Figs. 1, 2, and 3).

SUMMARY.

We have not found in the literature a report of an instance of permanent homoio- or autotransplantation of the spleen, or of the probably closely related spleno- and hemolymph glands. Spleen autotransplants with considerable difficulty as compared with thyroid, parathyroid, ovary, or adrenal cortex. This may be due to its complex anatomical structure. An instance of a permanent autotransplant has been observed. None of our attempts to homoiotransplant it were successful beyond the usual taking and persistence for 2 or 3 weeks, common to all homoiografts. The successful permanent subcutaneous autotransplantation had all the morphological characteristics of a fully differentiated and functionally active spleen. This method of transplantation would seem to offer a means of learning more of the normal development, regeneration, and function of this complex tissue.

EXPLANATION OF PLATE 54.

FIG. 1. Photomicrograph of the 325 day spleen transplant (Rabbit 14), showing adjacent abdominal muscle, capsule, and general characteristics. × 20.

FIG. 2. Photomicrograph of the same transplant, showing the structure. × 100.

FIG. 3. Photomicrograph of the same transplant, showing trabeculæ, pulp, and a Malpighian corpuscle. × 100.

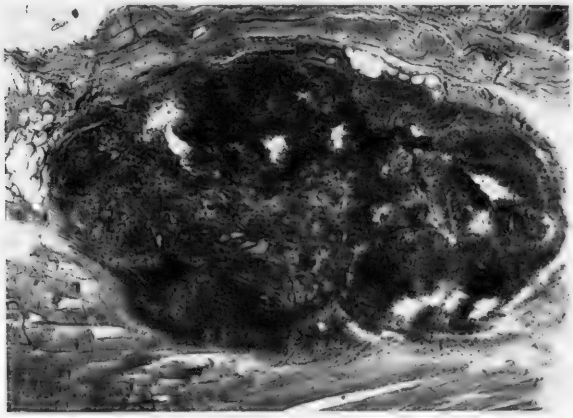

FIG. 1.

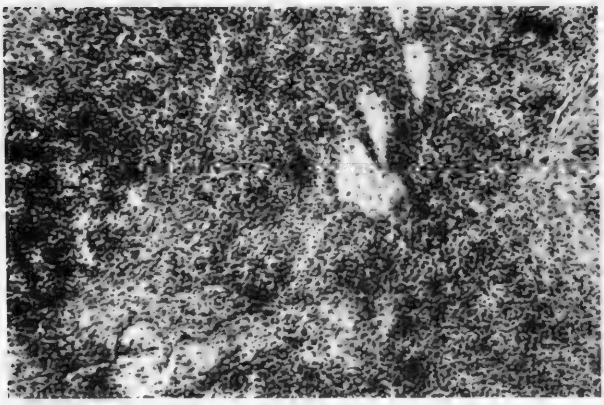

FIG. 2.

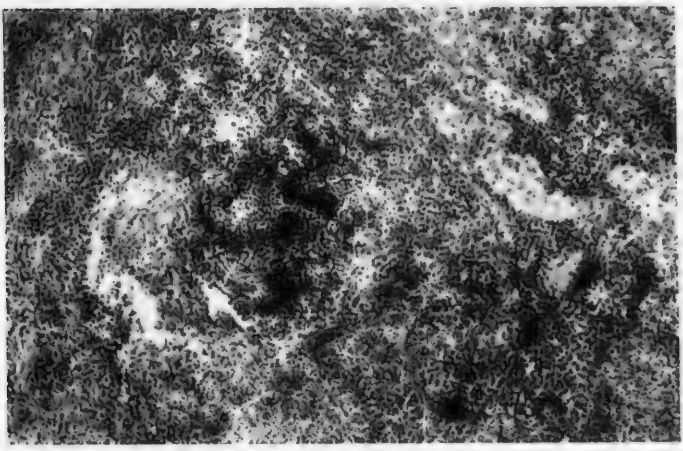

FIG. 3.

(Manley and Marine: Transplantation of Splenic Tissue.)

A NOTE ON THE PRODUCTION OF ANTIPNEUMOCOCCUS SERA.

By AUGUSTUS B. WADSWORTH, M.D., and MARY BUTLER KIRKBRIDE.

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(Received for publication, January 5, 1917.)

The value of homologous antipneumococcus serum as a therapeutic agent in lobar pneumonia caused by Type I pneumococci, has been demonstrated at The Rockefeller Institute Hospital during the past 3 years. It is desirable that the serum should be brought within reach of a larger section of the community than has been possible hitherto, but difficulties in production and administration of the serum have made this impossible. The present report deals entirely with the production of antipneumococcus sera.

In August, 1915, immunization of horses to pneumococci of the Types I, II, and III was commenced at the farm of the New York State Department of Health near Albany, under unfavorable conditions owing to inadequate facilities at the laboratory and the farm, and to irregularities in the injections caused by bad and occasionally impassable roads and by exposure of cultures to intense cold during the winter months. None of the horses immunized was under 11 years old. It was therefore thought that if potent sera could be produced under these adverse conditions, the success would strengthen the belief in these methods of immunization, and suggest possibilities for their broader application.

For immunization the method of Cole[1] with washed culture sediment and the method of Wadsworth,[2] using whole fresh 18 hour cultures, were followed. Beginning with weekly injections, or injections on 3 successive days each week, of heated cells or cultures, these were later followed by increasing doses of living organisms.

[1] Cole, R., *J. Exp. Med.*, 1912, xvi, 644.

[2] Wadsworth, A. B., *J. Exp. Med.*, 1912, xvi, 54, 78.

The potency of the sera was tested by protection tests on mice and by agglutination reactions. Intraperitoneal injection of control mice with 0.000001 cc. of the standard strains of Type I, II, or III, received from The Rockefeller Institute Hospital, and used for immunization and for these tests, was invariably fatal in less than 40 hours. Of three horses immunized against Type I organisms, 0.1 cc. of serum from No. 26 (culture sediment) or No. 28 (whole culture) protected, as a rule, against 0.4 cc., and not infrequently against 0.5 cc. of homologous culture. In three tests where 0.6 cc. of culture was used, the mice did not survive. 0.1 cc. of serum from No. 27 (culture sediment) protected, as a rule, against 0.2 cc., and sometimes against 0.3 cc. of homologous culture.[3] Wide variations similar to those reported by other workers were shown in the protection tests with Type II sera. 0.1 cc. of serum from No. 16 (culture sediment) or from No. 32 (whole culture) protected at times against 0.1 and 0.01 cc. of culture, and quite constantly against 0.001 cc.; a slightly lower protective titer was given by serum from Horse 29 (culture sediment).

Agglutination reactions indicated an inverse relation between the agglutinating power and the protective value of the sera, as in each type the horses with a lower protective index gave serum having a higher agglutination titer. Owing to the wide range of variations in agglutination reactions, due to differences in the density of the pneumococcus suspensions, satisfactory comparison with previous agglutination tests made by other workers is practically impossible.

While successful active immunization of rabbits, sheep, and goats with Type III organisms (*Pneumococcus mucosus*) has been reported, the production of sera of sufficient potency to agglutinate fresh untreated homologous organisms or to confer passive immunity on mice has apparently been unsuccessful hitherto. These failures have been considered to be due to the large mucoid capsule and tenacious slimy material produced by and surrounding the organ-

[3] The serum from Horse 27 has since protected 0.1 cc. against 0.4 cc. of culture. Experimental tests of different methods for standardizing the potency of antipneumococcus sera are in progress. Meanwhile in our experience more satisfactory results are obtained when 0.1 cc. of Type I serum instead of 0.2 cc. is used in protection tests on mice.

isms, which necessitated preliminary treatment, such as with a weak acid, according to the method of Porges,[4] before agglutinations could be obtained in the homologous sera.

For purposes of study and comparison, the immunization of a horse to Type III organisms (culture sediment) was carried on in conjunction with the horses of Types I and II.[5] The strain used for immunization while at first less virulent soon reached that of the other standard types, 0.000001 cc. killing a mouse within 40 hours. Protection tests showed that the serum possessed a definite though slight and variable protective action for mice. 0.2 cc. of serum in one instance protected against 0.001 cc. of culture, while in other tests mice receiving the same amounts of serum and culture died in 35, 63, or 70 hours. The same tests with several other Type III strains gave similar results.

In agglutination tests with fresh untreated Type III organisms, in concentrations of 1:1, agglutination was prompt and striking, usually showing before the tubes were placed in the water bath. Within 15 to 30 minutes agglutination was generally complete, and a loose cap had formed similar to those often seen in tests with Type I and Type II organisms and their homologous cultures. While agglutination in concentrations of 1:1 and 1:10 occurred as promptly as with Type I and Type II in their homologous sera, it was entirely absent in dilutions above 1:40. Undiluted serum obtained $5\frac{1}{2}$ months after the last immunizing injection agglutinated Type III organisms with equal promptness; serum of 1 month later, however, caused no reaction. This is of interest because the abscess which had been discharging during the previous six months had healed meanwhile. All strains of the *mucosus* type tested, which were morphologically and culturally typical, agglutinated promptly with

[4] Porges, O., *Wien. klin. Woch.*, 1905, xviii, 691. von Eisler, M., and Porges, O., *Centr. Bakteriol., Ite Abt., Orig.*, 1906, xlii, 660. Hanes, F. M., *J. Exp. Med.*, 1914, xix, 38.

[5] During the course of immunization a large abscess formed at the site of injection from which Type III organisms were isolated. Later a second abscess of a similar nature developed between the shoulder blades. It would be of interest to know the connection, if any, between these local foci of infection and the exceptional potency which the serum later developed.

one exception, but several strains which did not dissolve in bile failed to agglutinate. In a number of instances Type III organisms, which might readily have been classified as Type IV, owing to apparent absence in inoculated mice of the characteristic sticky peritoneal exudate and to the unusually small capsule of the organisms, were agglutinated promptly in Type III serum. Conversely, absence of agglutination in Type III serum made possible prompt exclusion from the Type III group of pneumococci obtained from the peritoneum of mice, in which a slightly viscous exudate was apparently present. The serum is, therefore, of distinct value in the diagnosis of pneumococcus types and should be used as a routine procedure together with the sera of Types I and II.

SUMMARY.

Horses immunized to Type I pneumococci developed serum, 0.1 cc. of which protected against 0.5 cc. of a virulent culture, 0.000001 cc. of which killed mice in less than 40 hours. Protective tests of serum from horses immunized to Type II organisms varied, 0.1 cc. protecting, however, in certain instances against 0.1 and 0.01 cc. of virulent homologous culture. Types I and II sera obtained in our experiments with culture sediment and whole culture did not vary markedly for a given type and corresponded closely in their protective titer with samples of sera received from The Rockefeller Institute Hospital. It is therefore evident that the following minimum standard of 0.1 cc. of serum to protect mice against at least 0.2 cc. of virulent cultures can and should be maintained when serum is to be used for the treatment of cases. By further study and comparison of these different methods of immunization it is hoped that sera of greater potency may be produced, but as yet this has only been accomplished in exceptional instances.

A horse immunized with Type III (*Pneumococcus mucosus*) developed serum having a slight degree of protection for mice against the corresponding organisms. This serum was sufficiently potent, however, to cause prompt and complete agglutination when combined with fresh untreated homologous organisms, thus avoiding the preliminary treatment to remove the capsule which has previously been held necessary. As a diagnostic aid in the differentiation of pneumococcus strains, the serum has proved of distinct value.

EXPERIMENTAL STUDIES UPON LYMPHOCYTES.

I. The Reactions of Lymphocytes under Various Experimental Conditions.

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(Received for publication, January 6, 1917.)

Hunger, chronic inanition, infections, or such specialized forms of injury as those caused by the x-ray, all bring about a sudden or gradual destruction of the small thymus cells. We have no knowledge as to the significance of the fragility of thymus lymphocytes, nor is anything known of the more intimate factors concerned. Compared with the detailed studies bearing upon the biological behavior of the red blood cells, our knowledge of the thymus cells and of the reactions of lymphocytes in general to environmental conditions is meager.

The fact that we have had no sharp criterion of injury, comparable to the hemolytic reaction, and available for comparative quantitative studies, is probably one of the reasons for this. Morphological changes in film preparations or tissues are often difficult to interpret. The cessation of ameboid activity, which has been used by Christian and Leen[1] and others as an index of cell injury, in the study of leukotoxins, is hardly applicable in the case of the sluggish and often entirely immobile lymphoid cell.

Since the introduction into common use of trypan blue and other vital stains, it has been repeatedly observed that the injured or dead cell reacts to these dyes by a diffuse staining of the entire cell, including the nucleus. Advantage has been taken of this fact by Gross[2] in his work on experimental nephritis, by

[1] Christian, H. A., and Leen, T. F., Some Further Observations on Leucocytotoxins, *Boston Med. and Surg. J.*, 1905, clii, 397.

[2] Gross, W., Experimentelle Untersuchungen über den Zusammenhang zwischen histologischen Veränderungen und Funktionsstörungen der Nieren, *Beitr. path. Anat. u. allg. Path.*, 1911, li, 528.

MacCurdy and Evans[3] in their studies of the cytopathology of experimental poliomyelitis, and by Rous and Jones[4] in their work on the protection of pathogenic organisms by living tissue cells. Evans and Schulemann[5] state that mechanical injury to leukocytes suspended in trypan blue causes instant diffusion of the dye into the traumatized cells.

It occurred to us that use could be made of this diffuse staining of injured cells to test the effect of various agencies upon the lymphocytes *in vitro*. A simple and satisfactory technique has been developed to this end. Our studies have been concerned chiefly with the small thymus cells of the rat, which provide readily available material, but we have applied the method also to lymphocytes from freshly removed human tonsils.

The method of procedure is as follows: The freshly excised thymus is teased with small forceps, the rat having been previously exsanguinated by aspirating blood from the heart, under ether anesthesia. There is obtained in this way a milky suspension composed entirely of small thymus lymphocytes, and practically free of red blood cells and fixed reticular elements. Suspensions of tonsil lymphocytes are prepared in a similar way, although it is more difficult to obtain them wholly blood-free.

The cell suspensions are then subjected to various experimental influences, and after a fixed period, a solution of trypan blue in Locke's solution is added in known dilution, which, however, may be purposely varied to meet different conditions. When the cells were suspended in Locke's fluid, a dilution of 1: 10,000 gave the best results; in the presence of serum or other colloids, a dilution of 1: 5,000 is preferable. A drop of the stained suspension is then placed in a counting chamber, and the percentage of stained cells immediately determined by counting from 300 to 600 cells with the low power.

There is generally no difficulty in detecting with No. 3 objective the sharply stained, non-refractile blue cells from the highly refrac-

[3] MacCurdy, J. T., and Evans, H. M., Experimentelle Läsionen des Centralnervensystems, untersucht mit Hilfe der vitalen Färbung, *Berl. klin. Woch.*, 1912, xlix, 1695.

[4] Rous, P., and Jones, F. S., The Protection of Pathogenic Microorganisms by Living Tissue Cells, *J. Exp. Med.*, 1916, xxiii, 601.

[5] Evans, H. M., and Schulemann, W., The Action of Vital Stains Belonging to the Benzidine Group, *Science*, 1914, xxxix, 443.

tile unstained lymphocytes. Under certain conditions, however, various degrees of transitional staining are encountered which make the determinations uncertain. This is particularly the case when the proportion of stained cells is high. There is then a tendency for all the cells to become gradually stained, so that a repetition of the count may give a much higher proportion of stained cells. On the other hand, when the proportion of stained cells is low, the time element becomes a less important factor.

Although these variations are at times disturbing and make it difficult to obtain coherent results, the method has proven of service, and by following a routine mode of procedure, the accidental fluctuations have been greatly reduced. We have repeatedly obtained duplicate counts varying less than 1 per cent, and in general the variations inherent in the method probably do not exceed 5 per cent, except when the proportion of stained cells is high.

Using this technique, we have systematically studied the reactions of the thymus cells under a fairly wide range of experimental conditions. The primary purpose was to test the availability of the method, so that our observations in many cases are merely the starting-point for a more thorough study of the questions involved.

The Effect of Hypertonic and Hypotonic Solutions.

Suspensions of thymus and tonsil lymphocytes were exposed to salt solutions of various concentrations, and the percentage of stained cells after the addition of trypan blue was determined. Locke's fluid was rendered hypotonic by the addition of distilled water, and the same test was applied.

The optimum concentration was found to be between 0.8 and 0.9, an increase in the percentage of stained cells being noted both with hyper- and hypotonic solutions. Hypotonic solutions of Locke's fluid were better borne than corresponding dilutions of pure sodium chloride.

The Effect of Varying Hydrogen Ion Concentration.

Fragments of thymus from the anesthetized rat were teased in balanced solutions of 0.093 M Na_2HPO_4 and KH_2PO_4, the hydrogen

ion concentration being varied from P_H 8.0 to P_H 6.0. The isotonicity of the solutions was tested against rat blood corpuscles. The percentage of stained cells upon adding trypan blue was determined after 1 to 2 hours. The time factor was checked, as far as possible, by counting the extremes of the series first, so that variations due to the longer exposure would affect especially the cells in solutions of approximately the hydrogen ion concentration of the blood.

Three repetitions of the experiment gave substantially the same result. The curves obtained, while not mathematically exact, probably represent fairly well the actual conditions. The optimum hydrogen ion concentration, or the concentration at which the minimum number of cells show diffuse staining, lies between P_H 6.8 and P_H 7.2. The variations between P_H 7.0 and P_H 8.0 are very slight, so we may infer that the fluctuations occurring during life in the circulating blood do not greatly affect the thymus cells. It is interesting to note that the optimum concentration, P_H 6.8 to P_H 7.2, is decidedly more acid than that of the circulating blood, but corresponds with the hydrogen ion concentration of the tissues as estimated by Michaelis.[6]

Beyond P_H 6.8, there occurred in all the experiments a rapid increase in the percentage of stained cells. At P_H 6.0 or P_H 6.2, practically all the cells stain diffusely. At the alkaline end of the series plasmolysis of the cells occurred and the counting of the cells became difficult. The rise in the percentage of stained cells is not so marked as with the acid solutions and was not obtained at all in one of the experiments.

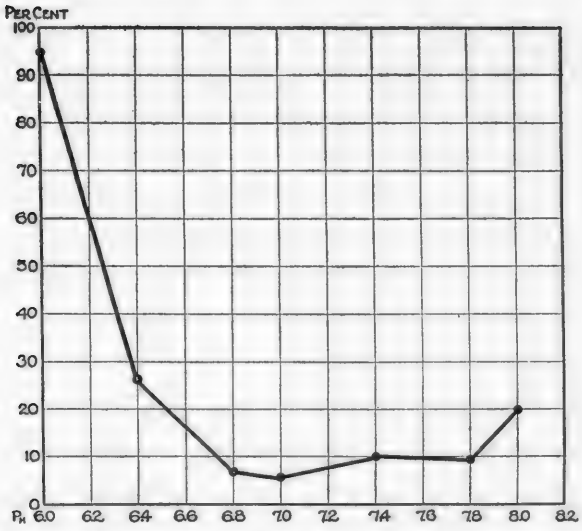
Text-figs. 1 and 2 are typical of the results obtained.

The Effect of Temperature.

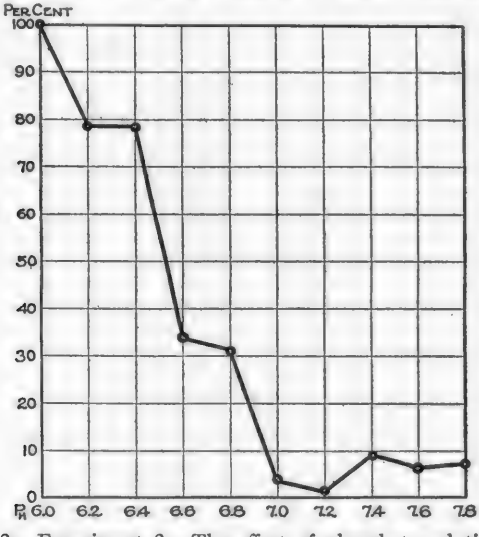
The effect of temperature was tested by immersing suspensions of thymus cells successively in small test-tubes in a water bath. Each tube was kept for 10 minutes at the desired temperature, and the proportion of stained cells immediately counted (Text-fig. 3).

It is seen that between 36° and 48°C. there is little variation in the percentage of stained cells. At 48°C. there is an abrupt rise in the curve, and at 51°C. practically all the cells are stained. The

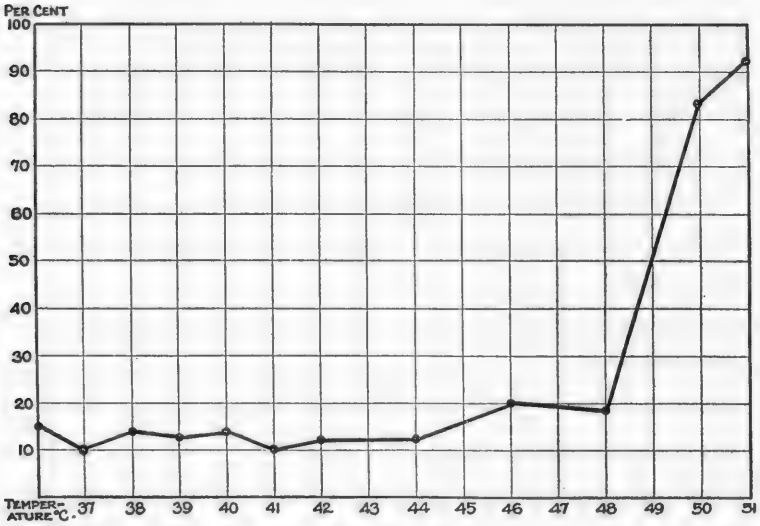
[6] Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914.



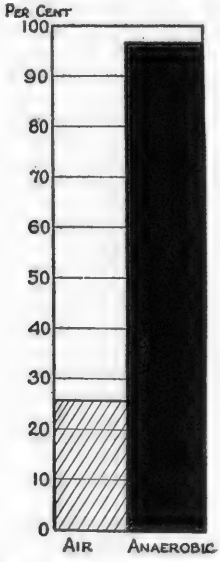
TEXT-FIG. 1. Experiment 1. The effect of phosphate solutions of varying hydrogen ion concentration upon the stainability of rat thymus cells.



TEXT-FIG. 2. Experiment 2. The effect of phosphate solutions of varying hydrogen ion concentration upon the stainability of rat thymus cells.



TEXT-FIG. 3. Experiment 3. The effect of temperature upon the stainability of rat thymus cells.



TEXT-FIG. 4. Experiment 4. The effect of asphyxia upon the stainability of rat thymus cells.

cells become irregular in shape and stain intensely. There is no diminution in their number. The curve suggests that the thermal death point lies between 48° and 51°C.

We have found no reference to the effect of heat upon lymphocytes, but Schultze[7] gives 50°C. as the temperature at which leukocytes (polynuclears?) undergo heat rigor.

Cells left over night in the ice box (temperature approximately 5–6°C.) show little if any increase in the proportion of stained cells. A detailed study of the effect of cold has not been made.

The Effect of Asphyxia.

Suspensions of rat cells were made in previously boiled Locke's solution, and covered with a layer of albolene. These were then placed in the thermostat, and compared with a similar suspension which had been exposed to the air. The asphyxia thus produced gives rise to a notable increase in the percentage of stained cells (Text-fig. 4). A similar effect was brought about by exhausting the oxygen with pyrogallic acid and sodium hydroxide.

If carbon dioxide gas is passed through a suspension of cells, flocculation occurs, the cells sinking in clumps to the bottom and leaving the supernatant fluid clear. The clumps, however, are easily broken up, and if the stream of carbon dioxide is suspended, the emulsion after being shaken resumes its normal appearance. The proportion of stained cells rises slowly, and is higher than that of a control suspension through which air is passed at about the same rate.

Pure oxygen passed through a suspension of cells brings about their rapid destruction. It was first thought that this might be due to trauma caused by the mechanical agitation; but the control in which air is allowed to bubble through vigorously shows that this is not the case.

The Protective Action of Serum and Other Colloids.

The addition of serum, even in relatively dilute amount, greatly lowers the percentage of stained cells, and in sufficient concentra-

[7] Schultze, M., quoted by Marchand, F., in Krehl, L., and Marchand, F., Handbuch der allgemeinen Pathologie, Leipsic, 1908, i, 50.

tion may completely inhibit the staining with the ordinary strength of trypan used. For this reason, in order to bring out differences where sera are being used, it is necessary to add trypan in a concentration of 1:5,000 or more.

The following experiment is an example of the inhibitory action of serum.

Tube.	Mixture.	Percentage of stained cells.
1	Locke's solution, 0.5 cc. Thymus cells, 3 gtt.	20.6
2	Locke's solution, 0.5 cc. Thymus cells, 3 gtt. Rat serum, 3 "	None.

Various organic and inorganic colloids were tested as to their ability to prevent staining. It was found that gelatin (3 gtt. of a 10 per cent solution) completely inhibited the penetration of the dye. Egg albumin conferred slight protection; starch and gum arabic were without effect in the concentrations tried. Hemoglobin obtained by laking washed cells in distilled water and rendering the solution isotonic with sodium chloride was also found to be inert. A cholesterol emulsion was made by dissolving pure cholesterol in acetone, pouring the solution into distilled water, and removing the acetone by boiling. The opalescent fluid showed no visible particles. After being made isotonic with sodium chloride, a thick suspension of thymus cells was added and the stainability of the cells compared with a suspension in pure salt solution. No protective action on the part of the cholesterol was observed.

The inorganic colloids tested were arsenious sulfide and colloidal iron. The former in stronger concentrations caused agglutination of the cells and was precipitated about them. In weaker dilutions it appeared to act like serum and gelatin in affording protection against staining.

Colloidal iron was precipitated and the cells were agglutinated in all the concentrations tried.

Extraction of serum with chloroform does not affect its protective powers.

The Effect of Certain Photodynamic Substances.

Hematoporphyrin.—The solution contained 1 mg. per 1 cc. dissolved in 95 per cent alcohol.

The toxic effect of this substance, when activated by direct sunlight, is shown in the following experiment.

Tube.	Mixture.	Treatment.	Percentage of stained cells.
1	Locke's solution, 1.0 cc. Thymus cells, 10 gtt.	Sunlight, 1 hr.	19.0
2	0.10 per cent ethyl alcohol in Locke's solution, 1.0 cc. Thymus cells, 10 gtt.	" 1 "	15.6
3	Hematoporphyrin, 1 : 100,000 in Locke's solution, 1.0 cc. Thymus cells, 10 gtt.	Darkness, 1 "	15.0
4	Hematoporphyrin, 1 : 100,000 in Locke's solution, 1.0 cc. Thymus cells, 10 gtt.	Sunlight, 1 "	99+

The cells which had been exposed to the action of hematoporphyrin in direct sunlight, in a dilution of 1 : 100,000[8] showed immediate staining upon the addition of trypan blue. The effect of sunlight alone or of the alcohol is negative, as shown in Tubes 1 and 2.

Chlorophyll.—An impure solution of chlorophyll, obtained by extracting cabbage leaves with 95 per cent ethyl alcohol, and diluted 1 : 100 with Locke's solution, was tested against rat thymus cells as follows:

Tube.	Mixture.	Treatment.	Percentage of stained cells.
1	1 per cent chlorophyll in Locke's solution, 1.0 cc. Thymus cells, 6 gtt.	Darkness, 30 min.	26
2	1 per cent chlorophyll in Locke's solution, 1.0 cc. Thymus cells, 6 gtt.	Arc-light, 30 "	68

Exposure of the serum alone to the action of these photodynamic substances did not impair its subsequent protective power for the cells.

[8] The same solution of hematoporphyrin causes complete hemolysis of washed rat erythrocytes in 1 : 100,000 dilution, the controls kept in the dark remaining unlaked.

The Effect of Exposure to X-Rays upon the Stainability of the Lymphocytes.

Because of the known susceptibility of the thymus cells to x-rays, we tested the value of trypan as an indicator of cell injury by exposing small tubes containing suspensions of cells to radiation.[9] The control tubes were unexposed, and we were not informed until the end of the experiment which of the tubes had been subjected to the x-rays.

No significant differences could be detected as regards the number of stained cells in the tubes which had been irradiated and the controls. In one of the experiments hourly counts were continued for 7 hours, at which time the percentage of stained cells had risen in both tubes so as to make the counts unreliable.

That this seemingly negative result was probably due to the existence of a latent period is indicated by the following experiment.

Experiment 5.—Two healthy young rats of approximately equal weight and age were etherized, and a small portion of thymus was resected. Cell suspensions in Locke's solution were immediately counted with the addition of trypan blue.

Rat 1 gave 5.9 per cent of stained cells.
" 2 " 6.3 " " " " "

This showed that the thymus cells of the two rats before exposure were almost identical in their reaction to the stain.

3 days later, the wounds being uninfected and the animals apparently in good condition, Rat 2 was given a 15 minute exposure to the x-rays. 24 hours later both animals were killed, and the suspension of thymus cells in Locke's solution was counted in the usual way.

Rat 1 (control) gave 10 per cent of stained cells.
" 2 (x-rayed) " 23.5 " " " " "

The difference in the proportion of stained cells, however, does not fully indicate the severity of the injury. The cells of the x-rayed rat were in all stages of fragmentation, and accurate counting was impossible. It was observed that many of the chromatin particles, which made up the bulk of the suspended material, showed great resistance to the staining with trypan. This would indicate that not every form of cell injury is associated with increased permeability to the dyestuff.

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[9] Dr. M. J. Sittenfield kindly assisted me in this work.

We made a number of experiments to determine whether exposure of the serum to the x-ray in any way altered its protective power, and with one unexplained exception we obtained only negative results. It would appear therefore that the inhibitory property of the serum is not affected, at least during the first 6 hours. The possible existence of a latent period has not yet been tested.

A Comparison of the Thymus Lymphocytes in Old and Young Animals.

In view of the involutional changes which occur in the thymus, and less strikingly in the lymphatic tissues generally with advancing age, we compared with this technique the behavior of cells of old and of young animals. The increased resistance of the cells of young animals has been evident throughout the course of the work, but several experiments have been made to test this point specifically.

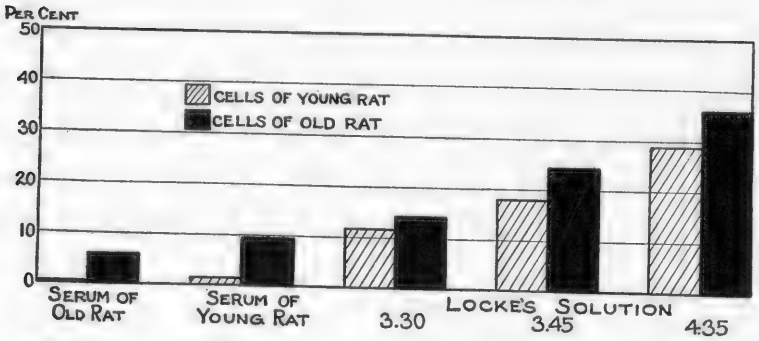
Experiment 6.—Suspensions were made from the thymus of a young rat and of a full grown adult animal. The cells were centrifuged and washed in two changes of Locke's solution to eliminate any possible action of the blood serum. The stainability of the cells was then compared in the usual way.

	Percentage of stained cells.		
Time	3.25 to 3.30	3.40 to 3.48	4.35 to 4.42
Young rat	11.1	18.2	28.7
Old rat	14.4	24.2	36.9

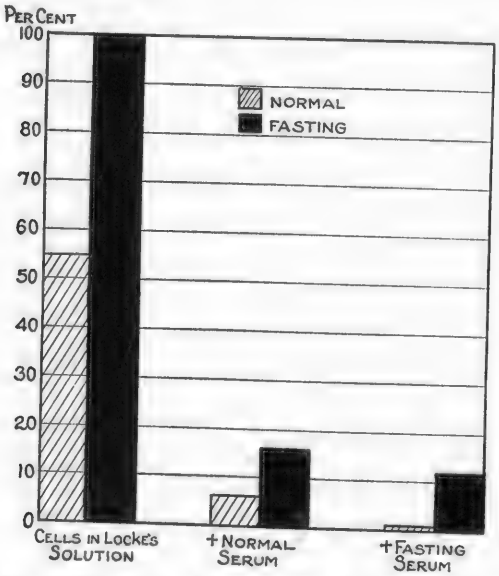
Several similar experiments gave identical results, so that there appears to be no doubt of the increased sensitiveness of the cells of the older animals to the unfavorable environment to which they are subjected under the conditions of the experiment. It will be interesting to extend this comparison to a study of the relative resistance to specific injuries of various kinds.

Fiore and Franchetti[10] in 1914 reported that they had produced premature involutional changes in rats by the injection of serum

[10] Fiore, G., and Franchetti, U., Su di una particolare proprietà del siero di sangue studiata in rapporto all'accrescimento dell'organismo è all'evoluzione del timo, *Atti Accad. med.-fis. fiorent.*, 1914, 87.



TEXT-FIG. 5. Experiment 6. The comparative stainability of thymus cells of young and of old rats.



TEXT-FIG. 6. Experiment 7. A comparison of the thymus cells and serum from normal and fasting rats.

from adult animals; and conversely, that the injection of "young" serum into old rats exercised a stimulating effect upon the growth of the thymus. This was contrary to our experience in as far as we had found the plasma of old animals a favorable medium for the growth of thymus tissue *in vitro*. In accord with other workers, we had found that the tissue of young animals showed a more vigorous growth than that of old.

However, the possible toxicity of the serum of old animals as compared with young ones was tested by subjecting young and old cells to the action of young and old serum. The comparative counts made with the usual technique do not indicate a toxic action on the part of the serum from old animals. When equal amounts of either young or old serum are added, there is always an increased stainability of the cells of the old animal. This is shown in Text-fig. 5.

The Effect of Acute and Chronic Inanition upon the Stainability of the Thymus Lymphocytes.

Perhaps the most striking feature in the pathology of the thymus is the atrophy brought about by acute starvation or prolonged inanition. This atrophy is due to the degeneration and partial disappearance of the small thymus cells, and leads, especially in the more protracted cases, to the most extreme reduction in the size and weight of the gland. The hunger involution has been repeatedly studied, both in the human subject, and experimentally by Jonson[11] and by Levin[12] in animals.

The following experiment, which has been performed several times with comparable results indicates that acute starvation brings about a greatly increased stainability of the thymus lymphocytes.

Experiment 7.—Rat 1, weighing 80 gm., was given water only for 3 days, during which period it lost 20 gm. in weight. It was then killed, serum was obtained and a suspension of thymus cells, twice centrifuged and washed, compared with a similar suspension from Rat 2, a control fed daily. Although this control rat

[11] Jonson, A., Studien über Thymusinvolution; die akzidentelle Involution bei Hunger, *Arch. mikr. Anat.*, 1909, lxxiii, 390.

[12] Levin, S., Recherches expérimentales sur l'involution du thymus, Thèse de Paris, 1912, abstracted in *Zentr. exp. Med.*, 1912, ii, 262.

was severely infected with *Trypanosoma lewisi*, and in consequence ill nourished and anemic, it showed a lower count than the fasting rat (Text-fig. 6).

	Mixture.	Percentage of stained cells.
Rat 1 (fasting)	Locke's solution, 0.5 cc. Thymus cells, 5 gtt.	100
" 2 (control)	Locke's solution, 0.5 cc. Thymus cells, 5 gtt.	54.3

The unusually high count in the control is probably caused by the chronic malnutrition. The average proportion of stained cells in a healthy young rat, as seen by reference to previous experiments, is from 5 to 15 per cent.

The following data exemplify further the regularly noted difference between healthy and ill nourished, diseased animals.

Experiment 8.

Healthy rat..... 8.7 per cent.
Emaciated rat..... 27.0 " "

The question arose as to whether the serum from a fasting or badly nourished rat contained substances toxic for the thymus cells of a normal animal. It was found, contrary to this idea, that the sera of fasting or emaciated rats conferred a greater protection against staining than did those of the normal controls, possibly because of the greater concentration of the sera under these conditions.

An experiment designed to bring out similar differences in the protective action of sera from normal and marantic infants, upon the stainability of human tonsillar lymphocytes, gave no significant results. The two sera also conferred identical protection upon rat thymus cells.

The Toxic Effect of Heterologous Sera.

That the toxic effect of an alien serum may be manifested in a higher proportion of stained cells is shown in the following experiment, in which the action of rat serum is compared with that of two human sera, upon rat thymus cells.

Experiment 9.

Rat cells—rat serum, 2 gtt	1.4 per cent.
" " —human serum (A), 2 gtt	9.2 " "
" " — " " (B), 2 "	9.4 " "

The toxicity of normal rabbit and guinea pig serum for rat thymus cells does not appear to be marked, although further studies are necessary. In one experiment the toxicity of horse serum was demonstrated, and also the fact that this toxicity, as judged by the increased percentage of stained cells, was not modified by heating to 56°C. for 15 minutes.

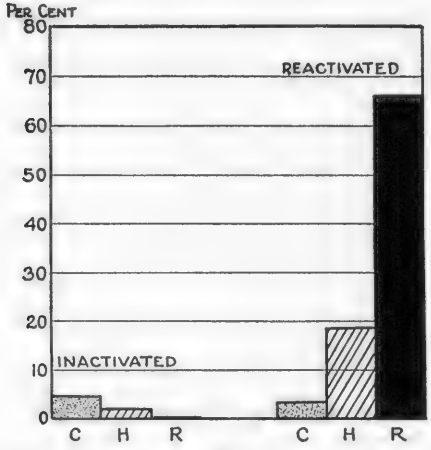
Specific Cytotoxic Serum.

A rabbit which had received four intravenous injections of 2 to 4 cc. of a thick suspension of rat thymus cells, has yielded a serum which is both agglutinative and cytotoxic. The agglutination is evident both micro- and macroscopically. The cytotoxic action is shown in the extreme morphological changes brought about in the cells in suspension (hydropic swelling of cytoplasm, pyknosis, karyolysis, etc.) and also sharply in the increased percentage of cells showing diffuse staining with trypan blue. Inactivation at 58–60°C. completely destroys the toxicity, and the addition of fresh guinea pig complement restores it. The agglutinin is not destroyed at this temperature.

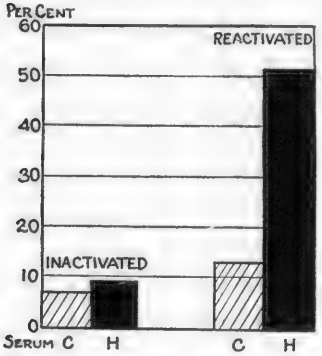
The serum is slightly hemolytic (1 : 10) for rat corpuscles and is also hemagglutinative. The hemolysin can be absorbed from inactivated serum by exposing it to washed thymus cells for 15 minutes at 37°C. On the other hand, exposure of the serum to red blood cells for the same period diminished, but did not wholly remove its thymotoxic action.

Text-figs. 7, 8, and 9 give some of the data upon which these statements are based.

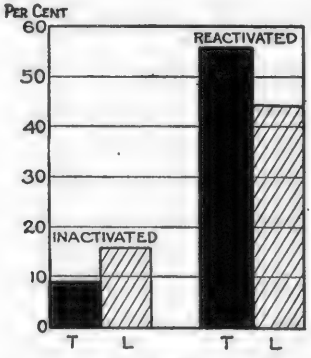
The serum of a rabbit immunized against human tonsil lymphocytes showed a slight toxicity for rat thymus cells as compared with the control normal rabbit serum. This suggests an organ-specific action upon lymphocytes, comparable with that established in the case of spermatoxin and lens precipitin. Further studies are necessary to determine this point. Against human tonsil lymphocytes,



TEXT-FIG. 7. Experiment 10. The effect of thymotoxic serum upon the stainability of rat thymus cells. *C*, control serum; *H*, serum of a rat immunized against human tonsil lymphocytes; *R*, serum of a rabbit immunized against rat thymus cells.



TEXT-FIG. 8. Experiment 11. The effect of lymphocytotoxic serum upon the stainability of human tonsil lymphocytes. *C*, control serum; *H*, serum of a rabbit immunized against human tonsil lymphocytes.



TEXT-FIG. 9. Experiment 12. The action of thymotoxic serum against lymphocytes from the thymus (*T*) and from a lymph gland (*L*).

the serum was highly toxic when reactivated with guinea pig complement. This is shown in Text-fig. 8.

Because of the discussion which has raged for many years over the question of the identity of the small thymus cells with the lymphocytes of other tissues, we undertook to test by this method the action of the thymotoxic serum upon the lymphocytes obtained from lymph glands.

Text-fig. 9 shows that both the agglutinative and cytotoxic actions are exerted equally upon lymphocytes of thymic and lymph gland origin. This seems to be strong evidence of their biological identity, more convincing perhaps than inferences based upon a common morphology.

CONCLUSIONS.

A simple method is presented by which, with the diffusion of trypan blue into the nucleus as a criterion of cell injury, it is possible to study quantitatively the effect of various agencies upon the small thymus cells and upon the tissue lymphocytes.

Preliminary studies with this method have led us to the following conclusions, which, however, unless otherwise stated, may be taken as applying only to the lymphocytes of the rat thymus.

1. The small thymus cells, when suspended in balanced phosphate solutions, show no distinct reaction to variations in hydrogen ion concentrations ranging between P_H 7.0 and P_H 7.8. Beyond P_H 7.0 there is a sudden increase in the permeability of the cells to the dye; plasmolysis of the cells occurs when the alkalinity exceeds P_H 8.0.

2. Heating to 49° or 50°C. is accompanied by a critical increase in the permeability of the cells to the dye.

3. The injury caused by lack of oxygen can be demonstrated by the increase in the number of stained cells.

4. The addition of serum to suspensions of thymus cells or tonsil lymphocytes greatly inhibits the diffusion of the trypan into the cells. The protection afforded is roughly proportionate to the amount of serum added.

Gelatin also exerts a marked protective influence; egg albumin affords a partial protection; starch and gum arabic are inert. Hemoglobin and cholesterol do not modify the stainability of the cells.

Arsenious sulfide in weak concentrations partially inhibits the diffusion of the dye. Colloidal iron is without effect, and is precipitated about the cells.

5. The toxicity of the photodynamic substance, hematoporphyrin, and of an impure chlorophyll solution in the presence of sunlight could be strikingly demonstrated by the greatly increased permeability of the cells to the stain.

6. Acute and chronic inanition produces an increased fragility of the cells. The protective power of the serum in acute starvation appears to be increased.

7. The small thymus cells of old animals are more readily injured than are those of young ones, as indicated by the increased proportion of stained cells.

8. The method has been applied to the demonstration of the action of cytotoxic immune sera for rat thymus cells and for human tonsil lymphocytes *in vitro*. Further experiments dealing with the question of specificity are in progress. The cytotoxins are inactivated by the addition of complement. Thermostabile cytagglutinins have also been produced.

I wish in conclusion to express my obligations to Miss Kate Brogan for her assistance in the technical part of the work.

THE NORMAL FATE OF ERYTHROCYTES.

I. The Findings in Healthy Animals.

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Plates 55 and 56.

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It has long been recognized that in the healthy body a considerable proportion of the erythrocytes are broken down and replaced every day—exactly what proportion is not known. The bile pigments have been deemed an indicator of the hemoglobin destroyed. Calculations based upon their rate of formation would seem to show that blood destruction is very rapid, in man from one-tenth to one-fifteenth of all the corpuscles being lost and replaced in 24 hours.[1] But the recent work of Whipple,[2] who has proved that the bile pigments may have other sources than the blood, demonstrates a large possible error in such calculations. Perhaps the most certain evidence of blood destruction is to be found in the constant activity of the bone marrow in the production of new cells.

The Phagocytosis of Red Cells.

There exists an extensive but inconclusive literature on the normal method of destruction of the red cells. Pearce and Austin[3] say truly that only two facts regarding this method have been established: first, that certain large endothelial cells, located for the most part in the spleen, take up red corpuscles and destroy them; and, second, that blood pigment is sometimes present in the Kupffer cells of the liver,

[1] Calculated from figures given by Howell, W. H., A Text-Book of Physiology for Medical Students and Physicians, Philadelphia, 1917, 6th edition.

[2] Whipple, G. H., and Hooper, C. W., *Am. J. Physiol.*, 1916, xl, 349.

[3] Pearce, R. M., and Austin, J. H., *J. Exp. Med.*, 1912, xvi, 780.

indicating that they may play a part in the destruction. Some investigators hold that phagocytosis is of itself sufficient to account for blood destruction. In our work, this possibility has been the first considered.

Certain cells of the bone marrow and, exceptionally, of the lymph glands may ingest red corpuscles, but they are in general negligible as compared with the phagocytes in the spleen. Sections of the spleen, as ordinarily prepared, contain so much blood as to conceal the phagocytes in large part and render difficult an estimation of their number. For this reason, we have examined sections of spleens washed out with Locke's solution by way of the vessels, and have searched the centrifuged washings as well. In order to prevent all clotting in the latter, they have been mixed with an equal bulk of an isotonic, watery solution of sodium citrate (3.8 per cent). The spleens of the guinea pig, dog, cat, rabbit, and monkey have been thus examined. That of the guinea pig contains many phagocytes. Numbers of them come away in the washings, a fact easily understood when sections of the half washed organ are studied, for it is then found to contain many wide venules or sinuses in which the majority of the phagocytes lie free mingled with the red cells. The spleen of the rabbit contains similar venules. Phagocytes may be few or plentiful in this animal, depending on individual variation; and not infrequently a few appear in the washings. The numerous phagocytes of the dog spleen are all retained during perfusion in the densely reticulated organ. The bone marrow of this animal may show many cells containing red corpuscles and pigment. The *rhesus* monkey, like man, has few phagocytes normally. The cat shows a practically complete lack of such cells. Indeed, in several adult cats, of which we examined the spleen, bone marrow, lymph glands, and other organs, they were exceedingly rare. It is evident that, in this species, blood destruction must take place by some method other than phagocytosis.

There are wide differences in the size of the spleens of the common laboratory animals, which denote as clearly as the findings just described the dangers of generalization from the results in a single species. In the rabbit, the spleen is so small as to be, relatively speaking, almost vestigial. We have found that it weighs on the

average about 0.6 gm. in an 1,800 gm. animal, thus constituting about 0.033 per cent of the body weight. In the rat, it is proportionally some eight times as large, about 0.27 per cent of the body weight,[4] and in the dog and cat, it represents 0.18 to 0.28 per cent[5] of this weight. These differences would be noteworthy were they dependent merely on differing functional demands of one sort. But the findings as regards phagocytes in the spleens of different species show that at least one function of the organ is subject to wide variation.

Some authors have suggested that the phagocytosis of red cells is not a vital phenomenon, but is agonal or occurs after death. That such a view is erroneous can be shown by opening the splenic vein of an anesthetized guinea pig, which has been washed almost free of blood by means of Locke's solution, introduced through the jugular vein and allowed to escape from the carotid artery. Under these circumstances, if the spleen is gently massaged, many phagocytes filled with intact and fragmented red cells come away in the splenic washings. As in the case of the other animal species examined, the phagocytes are much larger than the mononuclear cells of the circulating blood which take up red cells under pathological conditions, being from 45 to 60 μ or more in diameter. We have attempted experiments on the conditions governing the normal ingestion of red cells, using for this purpose phagocytes washed from the rabbit spleen, but they failed to survive more than a few minutes *in vitro*, as proven by the staining of their nuclei with trypan blue.[6]

The number of corpuscles ingested in the large spleens of the rat and dog is so considerable that, *a priori*, in the absence of definite knowledge of the rate of blood destruction, one might suppose phagocytosis to be the means whereby, in these creatures, the destruction of erythrocytes is accomplished. So, too, in the guinea pig. But in the cat, phagocytosis does not come in for consideration; and in normal man, the monkey, and the rabbit, it cannot be held responsible for the disappearance of any important amount of blood.

[4] Jackson, C. M., *Am. J. Anat.*, 1915, xviii, 75.

[5] This statement is based on isolated instances from several authors to whose papers Professor Jackson has kindly referred us.

[6] Evans, H. M., and Schulemann, W., *Science*, 1914, xxxix, 443. Rous, P., and Jones, F. S., *J. Exp. Med.*, 1916, xxiii, 601.

Extracellular Destruction.

The search for extracellular blood destruction was now begun. Cats were employed for the first experiments. After positive findings had been obtained with them, the observations were extended to other species.

From what is known of the resistance of the red cells, it would seem unlikely that their destruction takes place while they are still in circulation. Tests with hemolytic and hypotonic solutions according to the current methods fail to disclose signs that any of them are in process of breaking down. Some are more frail than others, but the resistance of all is considerable. The washed corpuscles of man and the cat may be shaken in bulk for a considerable time without the destruction of any. All this is difficult to reconcile with the idea that the cells circulate while disintegrating. It would seem either that they are removed by some organ, or set of organs, as soon as they begin to break down, or else that they leave the circulation even before this happens. In either case, one might expect to find somewhere in the body of animals in which phagocytosis is negligible, as, for example, in cats, a locus of disintegrating corpuscles. We have thought to find it by a search of the washings from organs perfused until entirely blood-free, combined with an examination of the washed organs themselves. Such a search presupposes a perfusion fluid in which the corpuscles will not be injured, and a method whereby those that are undergoing the normal disintegration may be collected from out the great mass of intact cells. The first of these requirements is fulfilled by the use of Locke's solution[7] containing a small amount of gelatin, and the second by differential centrifugation of the washings. When freed of serum and placed in normal salt solution, or Locke's or Ringer's solution, the erythrocytes of many species, especially those of the dog and of some rabbits, begin within a few hours to hemolyze. This, as one of us has shown, is the result of mechanical injury, entailed in even the most careful handling. It can be avoided by adding to the solution 0.125 to 0.25 per cent of gelatin.[8] In such a

[7] Locke's modification of Ringer's solution, but without sugar.

[8] Rous, P., and Turner, J. R., *J. Exp. Med.*, 1916, xxiii, 219.

fluid, delicate corpuscles remain intact, although pipetted or even shaken. The present work has constituted itself a severe test of the efficacy of the protection afforded by the gelatin. For the cell shadows that are the product of mechanical injury are easily recognizable, and by the differential centrifugation we have employed, they are brought into the preparations examined. Rarely has one been observed. Indeed, their consistent absence from the specimens obtained on perfusion and from teased preparations of the organs perfused renders it certain that normal blood destruction does not entail the formation of shadows.

We reasoned that disintegrating red cells would probably be lighter than sound ones because of some loss in substance or content. If this were true, they should come down last when the blood is slowly centrifuged in dilute suspension. It proved to be the case in preliminary tests with suspensions of the damaged cells of animals injected intravenously with a specific hemolysin.

Method.

A specimen of the animal's blood was taken by direct cardiac aspiration into a mixture of perfusion fluid and citrate solution, and then, under ether, the viscera were excised one at a time, after the vessels had been temporarily tied or clamped off, and immediately flushed out through the artery with Locke's solution containing 0.25 per cent of gelatin. A large gravity bottle was employed for the perfusion. Before it was begun, each organ was rinsed of surface blood, and during the process it lay in a porcelain dish into which the washings escaped by way of the natural venous channels. The perfusion pressure was ordinarily that of a column of the fluid some 120 cm. high. The washings which came last, and which were thought to represent, not so much the circulating as the residual blood, were caught separately and examined with special care. The kidneys, intestines, liver, and lungs were easily washed out. In order to wash the red bone marrow, a femur was excised, its surface flushed with the perfusion fluid, the bone was chipped away, and the marrow washed directly by means of hollow needles thrust repeatedly into it. In some cases, a limb of the animal was perfused *in situ*. Examination showed that the marrow then failed to wash out. The spleen and bone marrow were the only organs from which the blood failed to come away completely. In them a considerable trace always remained.

To prevent clotting the washings were caught in an equal bulk of isotonic, watery, sodium citrate solution (3.8 per cent). In the later work a mixture of equal parts of the citrate solution and gelatin-Locke's solution was used for the perfusion. 0.25 per cent of gelatin in the wash fluid was found to ensure protection in this ultimate mixture.

Portions of the washing were now slowly centrifuged in tall test-tubes and when the mass of red cells had come down, the shimmering, faintly pink, supernatant fluid was removed to another tube and sedimented at high speed. The slight sediment was suspended in a trace of fluid and examined microscopically. It consisted for the most part of platelets, red cells, and fragments of red cells.

The specific gravity of the wash fluid was such that no distinct leukocytic pellicle was formed.

Bodies Like Degenerated Red Cells.

Examinations of the organs of cats, rabbits, monkeys, and dogs, carried out according to this method, disclosed the presence of peculiar bodies in the washings from the spleen, bone marrow, and especially the liver, which yielded them in great number. They had the appearance of red cells and cell fragments which had lost their hemoglobin, but, unlike shadows, had retained something of their other substance, so that they were still refractile, though never markedly so (Fig. 1). They were not found in the washings from lung, limbs, or intestines. The kidney yielded a few of them. On centrifugation, they came down with the platelets, and when in great numbers, as in the liver washings, they tended on standing to collect into clumps (Fig. 1), which, however, were easily broken up with the pipette. They were spherical, oblong, or sausage-shaped, of all sizes up to that of the red cell or slightly larger, and of many degrees of refractility, some, especially the smaller, being difficult to perceive. The substance of the best preserved had often a greenish yellow cast, and what appeared to be transition forms from red corpuscles were easily found. When placed in blood serum, the appearance of the bodies did not change. Methylene blue and cresyl blue brought out in them a reticulum somewhat more delicate, as a rule, than that of reticulated red cells, but often indistinguishable from it (Fig. 2). Scharlach R and Lugol's solution failed to stain them.

Like red cells, the bodies were laked by water and by bile, acetic acid, saponin in high dilutions (1: 10,000), and by serum hemolysins when complement was present. In all these instances, shadows were left behind similar, except for the variation in size, to those of red cells. The bodies laked somewhat more easily than red cells, as was seen in preparations containing both. On standing in the ice box in salt solution, they became shadows after some hours or days. They

were crenated by hypertonic salt solutions, fixed by formalin and Cupp's fluid (Fig. 3), and agglutinated by specific agglutinins for the red cells. Elements morphologically indistinguishable from them were separated out by differential centrifugation from the blood of rabbits that had become anemic as the result of repeated injections of a serum hemolysin. We repeatedly recovered elements resembling them from the blood of patients with severe pernicious anemia, and from one case of congenital hemolytic jaundice. Other observers[9] have also found them in these conditions. They were regularly found in the circulation of rabbits engaged in breaking down a plethora of blood transfused to them from other rabbits. Furthermore, elements resembling them were observed within the endothelial phagocytes of rabbits injected intravenously with an hemolysin.

Despite all these characters which would seem to identify the bodies with disintegrating red corpuscles, they have another origin, being the products of injured cells of the fixed tissues. This can be shown by washing out at 5 minute intervals the liver of a rabbit, dog, or cat while it is still *in situ*. If ligation of the hepatic artery and the introduction of cannulas into the portal vein and the hepatic end of the inferior vena cava are carried out so rapidly that perfusion supervenes on normal circulation after the lapse of only 1 or 2 minutes, no bodies, or at most only a few will come away in the first washings which free the liver of blood. If there has been delay, the bodies will be found. Always they occur in great number in the washings obtained by perfusion after 5, 10, and 15 minutes. The result is the same whether the fluid used is a salt solution or defibrinated blood. In teased specimens of the liver and spleen, the cells of the parenchyma not infrequently may be seen to give off translucent globules, some of which when free constitute the bodies. Splenic phagocytes containing red corpuscles have been observed to give off "bodies" that are brightly tinted with hemoglobin.

If this is the origin of the bodies, how do they come to circulate in the blood of animals and human beings, the subjects of hemolytic processes? Those seen under such circumstances appear to have

[9] Lee, R. I., Minot, G. R., and Vincent, B., *J. Am. Med. Assn.*, 1916, lxvii, 719.

another source. They are probably reticulated red corpuscles, free of hemoglobin. Though morphologically such elements are characteristic "bodies," their reticulum as demonstrated with cresyl blue is moored in place, so to speak, as is true of reticulated cells in general, whereas that of the bodies usually draws together toward one side into a heavily pigmented skein or ball, some minutes after staining (Fig. 2).

The bodies differ in many ways from Albrecht's myelin, which is a relatively late postmortem product. A discussion of them at such length has seemed warranted because elsewhere we[10] have erroneously reported upon them as disintegrating red cells, and because they do indeed possess many characters which have been regarded as peculiar to erythrocytes. Their study should prove profitable to those interested in the constitution of the red cell.

In the subsequent work, each organ was washed out with all possible speed after the circulation had been interrupted. Often this was done with the organ *in situ*. As a result, the "bodies" have seldom been encountered.

Disintegration by Fragmentation.

The method just described has made possible a close search of the organs, one by one, for disintegrating red cells. Nowhere have hemolyzing cells been found; and the consistent absence from the washings of shadows, as already mentioned, is additional evidence that hemolysis in the gross sense does not occur. Instead, another and unsuspected method of blood destruction has been found; namely, disintegration of the cells, while they are still circulating, by fragmentation without loss of hemoglobin. It occurs in the cat, rabbit, and dog, all the animal species thus far examined.

If 2.5 cc. of blood is taken direct from the heart of a normal animal into 15 cc. of gelatin-Locke's-citrate and submitted to differential centrifugation, microcytes and poikilocytes similar to those of anemic bloods are regularly found, often in considerable numbers, among the platelets and other elements that are last to be thrown down. All stages in their derivation from red cells of normal size can be observed.

[10] Rous, P., and Robertson, O. H., Association of American Physicians, 1916.

Among the few of these latter that are associated with the small forms in the ultimate sediment, some are fragmenting, as shown by deep constrictions in their protoplasm or by their possession of slender processes from which small, hemoglobin-containing bits are separating off. The cells first thrown down by the centrifuge, that is to say the great bulk of the red cells, fail to show any such changes.

The division of red cells into microcytes and poikilocytes—schizocytes, as Ehrlich called them and as we shall for convenience refer to them—can be brought about *in vitro* by the pressure of a coverglass, or by gently heating the fresh preparation (Schultze, Rollet, Ranvier[11]). Such forms may also appear in the blood during caffeine poisoning and after prolonged anesthesia with chloroform.[11] The ease with which they are produced shows the need for careful controls to our observations. These have been carried out. Repeated manipulation of the diluted blood according to the method used in our work does not increase the number of fragmenting forms. They are found when the blood has been taken into oxalated salt solution instead of gelatin-Locke's-citrate. They occur in the blood from anesthetized animals. And, finally, they are present in undifferentiated fresh blood, though they are in general so few that it is easy to see why they have not been recognized as normally present there. They may easily be found in fresh rabbit blood. In looking for them care should be taken to use thick films of the blood, since in thin ones the cells tend to catch on the glass surfaces by which they are compressed and to be pulled in two as the drop spreads, thus producing an artificial fragmentation. This is avoided when the blood is examined in a thick layer or caught directly in a little gelatin-Locke's-citrate and then observed. Under both these circumstances, the number of fragmentation forms is usually small, and indeed some rabbit bloods fail to show them except on long search. In most bloods, though, they are regularly found, one to twenty being noted as a rule in a 5 minutes' search. In the individual the number is remarkably constant from day to day. It may be considerably increased when there is the least anemia.

In the washings from the organs, as obtained by our method,

[11] Cited in Krehl, L., and Marchand, F., Handbuch der allgemeinen Pathologie, Leipsic, 1908, i.

much normal blood is always present, and in consequence schizocytes are always found. It has been necessary to control this factor in the search for accumulations of disintegrating cells. Our practice has been to make in perfusion fluid suspensions of the animal's heart's blood of approximately the same hemoglobin content as each of the organ washings, and to differentiate them in the centrifuge with the latter. Such dilute specimens of the circulating blood yield few schizocytes, and any marked preponderance of them in the washings can with good reason be attributed to the organ's special content.

The results of our search of the organs can be stated briefly. The only evidence encountered of extracellular blood destruction lay in the presence, in the spleen especially, of accumulations of microcytes and poikilocytes such as had been met with in the circulating blood. The spleens of the dog, rabbit, and cat regularly contained numbers of these small forms. They often failed in large part to wash out, being then discoverable in the tissue teased after perfusion. Systematic observations in rabbits have shown that there may be small collections of them elsewhere, most frequently in the kidneys, but these are inconstant and always insignificant as compared with the splenic content, even when the difference in size of the organs is taken into account. The red bone marrow (femur and rib) is notably free of the forms. It is plain that they are not put forth as such by the marrow. Confirmatory evidence on this point is presented in our second paper.

The normal spleen may retain sufficient schizocytes after washing to give the organ a brownish red color, as in the case of many rabbits and cats, or this color may be due in greater part to phagocyted red cells, as in other rabbits and in dogs. In some healthy animals, certainly, the accumulation of schizocytes is not great. Some cat spleens can be washed almost white, and the washings in these cases may contain only moderate numbers of the fragmentation forms. Their number is moderate, too, in the large spleen of the dog. But they are always present and are much more frequent than in the circulating blood. The difficulty with which they are dislodged from the spleen, even on repeated perfusion, is striking. The first

washings in which most of the blood comes away contain but few of them.

A number of control tests have been made which prove that the splenic schizocytes are not artefacts. They are regularly found when the fresh organ is teased in autogenous blood serum. The teasing itself does not cause them, for other organs containing the same amount of blood and similarly teased fail to yield them. They have no relation to the use of ether as an anesthetic. The possibility that they are fragments of corpuscles extruded by phagocytes in animals possessing such cells has been ruled out by means of a differential stain. In fresh preparations in isotonic salt solution, to which a little cresyl blue has been added, the fragments of red corpuscles resulting from intraphagocytic digestion become a deep, opaque blue-black, whereas those produced by extracellular fragmentation fail to stain at all. The specificity of the dye is, in this regard, striking. Red cells when first phagocyted fail to stain. Later they stain copper-green or intensely blue. As breaking down proceeds, the affinity for the stain grows, and the intracellular fragments of red cells stain almost black in solutions of cresyl blue so weak as scarcely to affect other elements. In teased specimens, these blue-black fragments are not infrequently found lying free, a sharp contrast to the non-staining fragments (schizocytes) that result from extracellular destruction.

The Schizocytes.

The shape of the poikilocytes and microcytes found in the peripheral blood and the spleen is to some extent peculiar to the animal species. In the cat spherical or oblong forms predominate (Fig. 4); in the dog, short, blunt rods exist as well; in man, in addition to these, shapes like small asymmetrical starfish are observed; while in the rabbit, there is the greatest diversity of forms—spheres, capsules, shapes like drumsticks, rods, dumb-bells, pears, balls with strings to them, and even short, thick threads brightly tinted with hemoglobin (Fig. 5). If anything, the schizocytes are of a more intense orange hue than ordinary corpuscles. Many very small forms are seen. From the larger forms, fragments so minute as to be at the limit of microscopic visibility can be seen to break off. The

ultimate fate of all would seem to be division and redivision into a fine dust.

Red cells of approximately normal size, from which fragments are in process of separation, are fairly common in the spleens of all the species we have examined. As already stated, they are present in the ultimate sediment obtained on differential centrifugation of the circulating blood. A form always to be found in the rabbit spleen, and sometimes recovered from the blood by differential centrifugation, consists of two deeply tinted portions of corpuscle held together by a median zone of protoplasm so flattened anteroposteriorly as to appear devoid of hemoglobin (Fig. 6), except when viewed from the side. When so viewed this form has a dumb-bell shape, and the connecting zone is seen to be well provided with pigment. In the end the zone gives way, and the cell portions become separate microcytes. We have never found any indication of the decolorization of microcytes in the spleen. In the cat spleen there may sometimes be encountered a few pale and exceedingly thin corpuscles, still tinted with hemoglobin, which look as though they were dissolving away much as discs of sugar dissolve in water.

SUMMARY.

The phagocytosis of red corpuscles, while frequent in the normal dog, rat, and guinea pig, is slight in man, the *rhesus* monkey, and many rabbits. In cats it is always negligible in amount and frequently absent. Phagocytosis will not suffice as a general explanation of normal blood destruction.

When the liver, spleen, and bone marrow of the cat, dog, rabbit, or monkey are slowly perfused with defibrinated blood or Locke's solution, bodies are given off into the fluid which have the appearance of red corpuscles that have lost their hemoglobin but retained the rest of their cell substance. These bodies possess many of the properties supposedly distinctive of red corpuscles. They are the product of disordered parenchymal cells.

By a special method, it has proved possible to search the body, organ by organ, and the circulating blood also, for disintegrating red corpuscles. Shadows of red cells are not present anywhere, nor are hemolyzing red cells found. A hemolytic process, in the

ordinary sense of the term, can scarcely play an important part in normal blood destruction. Instead, it is certain that some red corpuscles, at least, are destroyed in another way; namely, by fragmentation. Normal blood regularly contains small numbers of fragmentation forms—microcytes and poikilocytes—and accumulations of them are regularly present in the spleen, but are found only inconstantly in the other organs. The fragments are in evident process of further subdivision. They occur not only in species in which phagocytosis as a means of cell destruction is negligible (cats), but also in animals in which it is an important process (dogs, some rabbits).

The method of study that we have employed is well suited to disclose how the blood is destroyed. The importance of cell fragmentation in this connection is indicated by our failure to find any other means of destruction, save only the phagocytosis already known. Further facts indicating the importance of fragmentation are presented in our second paper, where a general discussion will also be found.

EXPLANATION OF PLATES.

PLATE 55.

FIG. 1. "Bodies" from the liver washings of a dog. Five ordinary red cells and a leukocyte are also to be seen. The arrows point to the bodies, which are clumped as the result of centrifugation. Fresh preparation.

FIG. 2. "Bodies" stained with cresyl blue. A, immediately after staining; diffuse reticulum. B, later effect; the reticulum has massed to one side. From the liver washings of a dog.

PLATE 56.

FIG. 3. "Bodies" fixed with Cupp's fluid (a formalin-potassium-bichromate-acetic-acid mixture). A, bodies; B, red cells. From the liver washings of a monkey.

FIG. 4. Microcytes and poikilocytes collected by differential centrifugation from the blood of a normal cat. Wright's stain. The upper arrow points to a round microcyte, and the lower to a poorly focused oblong form.

FIG. 5. Microcytes and poikilocytes collected from the blood of a normal rabbit. The dark dots are platelets. The arrows point to the schizocytes of which the diversity of form is well shown. Wright's stain.

FIG. 6. Fragmenting red corpuscles from the spleen of a rabbit. Wright's stain. There is no real loss of hemoglobin from the pale portions of the cells, but here the layer of protoplasm is very thin.

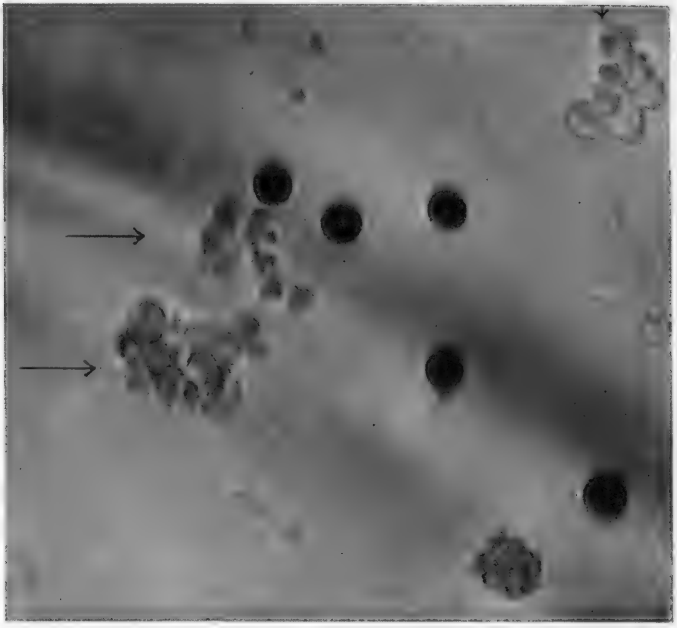

FIG. 1.

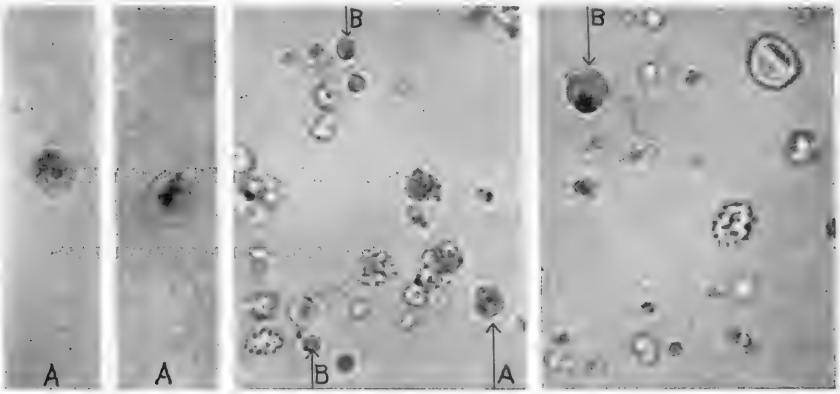

FIG. 2.

(Rous and Robertson: Normal Fate of Erythrocytes. I.)

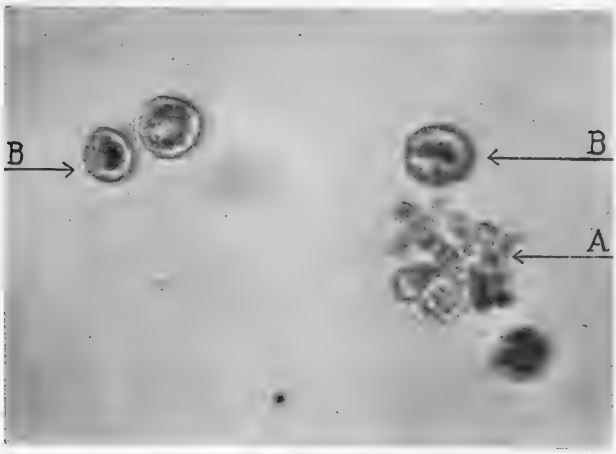

FIG. 3.

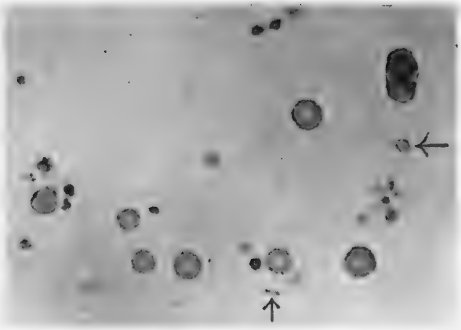

FIG. 4.

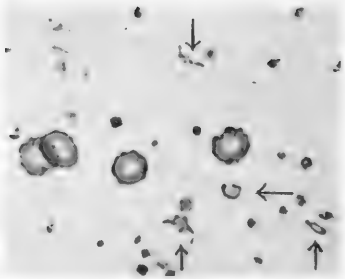

FIG. 5.

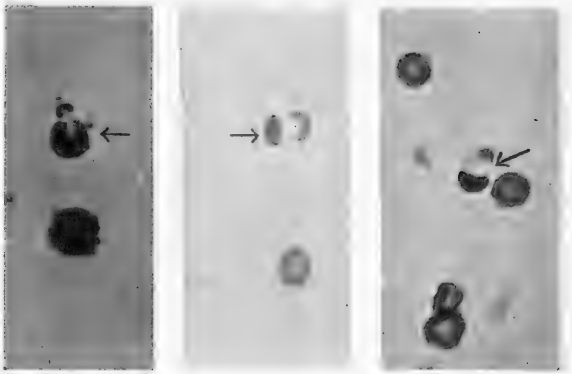

FIG. 6.

(Rous and Robertson: Normal Fate of Erythrocytes. I.)

THE NORMAL FATE OF ERYTHROCYTES.

II. Blood Destruction in Plethoric Animals and in Animals with a Simple Anemia.

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The findings presented in the preceding paper show clearly that phagocytosis can account for red cell destruction only in certain species, and that whatever the extracellular method of this destruction may be, it does not entail the formation of shadows, such as result from hemolysis. The evidence is against a direct hemolytic action in the spleen. The constant presence in this organ of an accumulation of poikilocytes which are subdividing, and of microcytes, and the presence of these elements in the circulating blood indicate that the red cells disappear, in part at least, by fragmentation. In a further study of methods of blood destruction, we have examined rabbits rendered plethoric by repeated direct transfusion. Such animals soon acquire the ability to dispose of large quantities of blood. This goes on in the absence of demonstrable agglutinins or hemolysins, and, according to Boycott and Douglas,[1] represents an intensification of the normal process of destruction, though what that is the authors cited could not discover. They state that the spleen is the only organ in which changes are regularly met with. It is enlarged, and phagocytes containing red cells are more numerous than usual.

Method.

Rabbits were used. From three to six compatible donors were selected for each recipient. The donors were employed in rotation, and each recipient was given nearly every day 10 cc. of whole blood from one of them. The blood was

[1] Boycott, A. E., and Douglas, C. G., *J. Path. and Bacteriol.*, 1909–10, xiv, 294.

obtained by cardiac aspiration into a syringe containing 1 cc. of 1 per cent sodium citrate in 0.9 per cent salt solution. It was injected directly into an ear vein of the recipient. Clotting was not met with. The small amount of citrate—0.01 gm. at each injection—caused no symptoms or lesions.[2] The transfusions were continued for several weeks, during which time the animals remained well and frequently gained in weight.

The rabbits were found to differ much in their ability to dispose of the excess blood. In some, it was difficult to increase the hemoglobin value even by daily injections; in others, it began at once to rise, and soon reached a high figure. Since the total blood volume from day to day was not known, it is impossible to say how much these differences are attributable to variations in the plasma. As a rule, when the animal had been transfused for some time, and the hemoglobin had increased from 80 or 90 per cent Sahli to about 150 per cent, it failed to go higher, but instead tended to drop despite the daily addition of blood. Yet very often in such animals, no demonstrable agglutinins had developed. These animals were judged suitable for our purpose. The circulating blood was examined for disintegrating red cells, the animal was etherized, and the organs were washed out one by one according to the methods outlined in our first paper. The washings were submitted to differential centrifugation, and portions of the organs were teased and examined.

The circulating blood of many of the plethoric animals showed microcytes and poikilocytes—schizocytes—in far greater numbers than did that of normal controls; but no other signs of blood destruction were seen in it. Examination of the donor rabbits showed that the increase in microcytes and poikilocytes was in general traceable to them. The removal from these animals of 10 cc. of blood every 4 or 5 days was often sufficient to cause a marked increase in the number of schizocytes in circulation, whence they were transferred to the plethoric individuals. When care was taken to use donors with few circulating schizocytes, it was found that the increased blood destruction in the plethoric rabbits was unaccompanied by an increase in these forms in the blood.

There was regularly found a notable increase in the number of schizocytes in some of the organs under circumstances which precluded an introduction of them with the donor's blood. For example, in one animal repeatedly transfused during 14 days, and always with a blood in which schizocytes were rare, the spleen was found to be packed with these forms. It was a large, brown-purple organ, from

[2] In control animals injected with sodium citrate alone, normal findings were obtained.

which several times as much blood as normal was obtained on perfusion. After washing, it was smaller than before, but still enlarged, weighing 1.1 gm. as compared with 0.6 gm. for the average normal individual of the same size. In the teased specimen phagocytosis was only slightly increased, but there were great numbers of microcytes, poikilocytes, and the peculiar dumb-bell form of fragmenting red cell encountered not infrequently in normal animals.[3] In fact, nearly all the considerable quantity of blood which failed to wash from the spleen consisted of these forms. In the washings they were far more frequent than is ever the case in normal blood. No agglutinated masses or shadows were observed.

This is a typical finding. In some spleens, small extracellular aggregates of amorphous brown pigment were frequent. Sometimes phagocytosis was increased, but often it was only normal. The one unfailing and characteristic feature was the great accumulation of schizocytes.

In the other organs, with the exception of the red bone marrow, the accumulation of schizocytes was inconstant and always negligible. The fact has already been brought out that the normal marrow contains only such schizocytes as happen to be present in the blood coursing through it. In the plethoric marrow, on the contrary, they are met in considerable numbers. Occasionally the number of phagocytes containing red cells is somewhat increased. We have utilized the peculiar affinity of the cresyl blue stain for intracellular red cells and their fragments[3] to determine whether the free schizocytes in marrow and spleen are the product of intraphagocytic digestion. This is not the case.

Despite careful search, no other methods of blood destruction were observed. The findings strongly support the view that the disappearance of red cells in plethoric rabbits takes place in large part by fragmentation, if not almost wholly.

Blood Destruction in Animals Anemic from Hemorrhage.

Microcytes and poikilocytes are frequent in the blood of animals rendered anemic by hemorrhage. The conception that these forms

[3] Described in the preceding paper.

are the result of blood destruction finds here an apparent contradiction; for in simple anemia one would expect a conservation of blood rather than increased destruction.

We have examined numerous anemic rabbits by perfusion and differentiation of the washings in the centrifuge, according to the method already described.[3] To render the animals anemic they were bled on successive days by cardiac aspiration, 25 cc. being the usual amount of blood removed. After four or five bleedings, when the hemoglobin had dropped to 30 to 20 per cent, no more blood was taken. 3 or 4 days after the last bleeding, films of the blood showed that repair was in progress. There were many reticulated corpuscles, numerous large pale ones, and, in addition, marked polychromasia and numerous microcytes and poikilocytes. It was at this stage that the animal was etherized and the organs examined.

The findings may be briefly summarized. There was a striking increase in the spleen's content in microcytes and poikilocytes. Much of its residual blood consisted of these forms, and, in several instances, the organ was somewhat enlarged from their accumulation. Phagocytosis was not increased. In some of the other organs, especially in the kidney, small collections of microcytes and poikilocytes were inconstantly present. It was noteworthy that the washings from the marrow never yielded more of them than did the control specimen of circulating blood, while in the marrow tissue teased after washing, there were practically none. The point had special attention because of the widely held view that these forms originate as such from the blood-forming tissue. Our findings show that this is not the case, and that the microcytes and poikilocytes of anemia from hemorrhage are true fragmentation forms, deserving of the name of schizocytes given to them by Ehrlich.

Several facts were observed which indicate that the schizocytes of anemia are derived for the most part not from the fragmentation of cells remaining from the pre-anemic period, but from the breaking down of cells formed by the bone marrow to make up the lack in blood. Most of the schizocytes of the anemic rabbits partook of the pallor of the new-formed corpuscles. In the centrifuge, they were readily separated from the mass of cells but were themselves completely sedimented only with difficulty; and the last

to come down from suspension were practically colorless. Large numbers of the schizocytes were reticulated like the new corpuscles circulating with them. In two instances in which counts were made, 56 and 25 per cent of the schizocytes were reticulated, as compared respectively with 30 and 24 per cent of the corpuscles, a relation readily understood when it is considered that one reticulated corpuscle might give rise to several reticulated schizocytes. In the normal rabbit, as is well known, 2 per cent of the corpuscles or less show reticulation, while according to our experience, none of the schizocytes are reticulated. In the anemic rabbit, reticulated schizocytes appear almost immediately after any great increase in reticulated corpuscles. The conclusion seems warranted that the schizocytes of the anemia result from the fragmentation of new-formed, abnormal red cells. Some of the latter must begin to break in pieces almost as soon as they are put forth by the marrow. From the study of a fresh preparation of anemic blood, some idea can be had of the ease with which this may occur. As the blood drop spreads under pressure of the cover-glass, it will be observed that the well colored, normal looking red cells constitute stable objects as compared with the pale, large cells resulting from the anemia. Often one of the latter will be swept through a narrow cranny between two normal cells. The contour of these latter does not change in the least, whereas the pale cell is greatly compressed, and it flows through the aperture as if fluid. It is easy to see how such cells may be pulled to pieces in the circulation.

Altogether, the findings in anemic animals indicate that here the destruction of red cells by fragmentation goes on actively. Large numbers of the fragments accumulate in the spleen. Many, though, circulate for a greater or less time. This is not the case with the schizocytes of normal and plethoric animals, which are rapidly taken out of the blood.

DISCUSSION.

Ehrlich long ago stated[4] that the microcytes and poikilocytes of anemia result from fragmentation of the circulating cells. He

[4] Ehrlich, P., Farbenanalytische Untersuchungen zur Histologie und Klinik des Blutes, Berlin, 1891, 99.

based his view on the fragmentation *in vitro* of red cells under the action of various physical and chemical agents. His conception is still far from having gained general acceptance, many hematologists holding the view that microcytes and poikilocytes are elaborated as such in the marrow.[5] Our findings show definitely that they arise in the circulation. Ehrlich thought that their appearance during an anemia was purposeful, since it increased the blood surface, and, as a corollary to this opinion, he held that they circulated for some time. Two of our facts point against the idea of a purposeful division. The cells which break down are those least suited to combat the anemia, namely, those poor in hemoglobin; and the fragments tend to accumulate in the spleen, whence they cannot easily be flushed out. Cell fragmentation in an anemic organism would seem to be, not the result of a compensatory arrangement, but an incident in a vicious circle. Because of the anemia, the bone marrow puts forth cells that are unable to withstand the exigencies of circulation. These soon fragment and the work of the marrow must be done again. The marrow then must not only make up an initial blood lack, but while so doing must repair constant fresh losses due to the poor quality of the cells it puts forth. Or, in other words, it must take one step down for every two steps up. That there is no increase in the output of bile pigments in anemic animals may be due to a special conservation of hemoglobin, or to the relative paucity of this substance in the broken down cells, or to a diminution in the breaking down of such normal red cells as are present, or to extraneous factors such as Whipple has shown can influence the pigment output.

The view that red cell destruction normally takes place for the most part by fragmentation finds striking support in the findings in both anemic and plethoric animals. In the former, the red cells are less hardy than usual. Fragmentation is the result. In the latter, a greater blood destruction than usual must be accomplished. Again fragmentation is increased. Furthermore, in our plethoric animals, which would seem especially suited to the investi-

[5] For the latest statement of this view see Barker, L. F., The Clinical Diagnosis of Internal Diseases, New York, 1916, iii, 25.

gation, no other method of destruction than fragmentation, saving only the phagocytosis already known to exist, has been encountered. Phagocytosis is often increased in these animals, but not always. In some cases, fragmentation is practically the only process of destruction found.

The accumulation of schizocytes in the spleen is striking, both in anemic and plethoric animals. That this organ has some important function in connection with such elements cannot be doubted; and the findings in plethoric animals suggest that the bone marrow may share the function if blood destruction is great. When human beings are severely burnt, many red cells break into hemoglobin-containing fragments. According to good authority,[6] these collect in the spleen so rapidly that within a few minutes practically all are removed from the blood. Not improbably the blood of normal and plethoric animals is kept free of fragments in much the same way. They are not taken so completely from the blood of anemic animals. Perhaps a change in the protoplasm, such as may be thought to accompany normal aging of the cell, is necessary for this. But even in anemia, microcytes and poikilocytes accumulate in the spleen to a noteworthy extent.

The schizocytes are undoubtedly reduced at last to a fine hemoglobin-containing dust. How this is disposed of, whether in a special organ, like the spleen, or by dissolution in the blood is not evident. But it must be disposed of rapidly, or the blood of plethoric animals would be rich in it. The hemoglobin, wherever and however given off, will of course be quickly utilized by the liver.

Taken together, the facts afford a simple and rational explanation of the normal method of blood destruction, and indicate why the problem has presented difficulties. A constant rapid fragmentation of the effete red cells, one by one, while still circulating, and a prompt utilization of the products of destruction will readily account for the high general standard of cell resistance that has puzzled observers. Whether, indeed, as the red cells fragment, their resistance lessens, as determined by the ordinary tests, remains to be determined. The

[6] Krehl, L., and Marchand, F., Handbuch der allgemeinen Pathologie, Leipsic, 1908, i.

fragmentation does not involve a loss of hemoglobin, and *a priori* there is no reason why it should be accompanied by a decreased resistance to hemolysins or hypotonic fluids, the ordinary test-agents.

Meltzer[7] has suggested that the red cells are normally destroyed through mechanical wear and tear, and has studied in this connection their fate when shaken. But cells shaken *in vitro* are not destroyed according to the method we have encountered in the body. Only a few of the shaken cells break into microcytes and poikilocytes. The vast majority are reduced to shadows. Shaking is obviously a different process from the perpetual sieving and squeezing of the cells that go on in the finer capillaries. Yet it is possible that the resistance of the corpuscles to shaking may after all give some indication of their tendency to fragment. Experiments to determine the point are now under way.

GENERAL CONCLUSIONS.

1. The increased destruction of red cells in animals rendered plethoric by transfusion takes place predominantly by a fragmentation of the corpuscles without loss of hemoglobin.

2. The microcytes and poikilocytes observed in animals with a severe anemia due to hemorrhage are not put forth as such by the bone marrow, but are portions of cells fragmented while circulating.

3. The cells thus fragmented are for the most part those newformed to meet the exigencies of the situation. Such cells are in large part unable to withstand the wear and tear of function. There results a vicious circle. The anemia renders the bone marrow unable to put forth proper cells, and those it does produce are soon destroyed, thus prolonging the condition. A similar state of affairs probably exists in many human anemias.

4. The occurrence of large accumulations of microcytes and poikilocytes in the spleen of anemic and plethoric animals indicates that the organ exercises some important function in connection with these forms. The same is true of normal animals, for the findings in them are similar, though less striking.

5. The normal fate of the red corpuscles, in those species in which

[7] Meltzer, S. J., *Rep. Johns Hopkins Hosp.* (*Welch Festschrift*), 1900, ix, 135.

phagocytosis is negligible, is to be fragmented one by one, while still circulating, to a fine, hemoglobin-containing dust. The cell fragments are rapidly removed from the blood, but their ultimate fate remains to be determined. The facts indicate that they are removed from the blood by the spleen, and under exceptional conditions, by the bone marrow.

THE ELIMINATION OF IRON AND ITS DISTRIBUTION IN THE LIVER AND SPLEEN IN EXPERIMENTAL ANEMIA.

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(Received for publication, December 20, 1916.)

It has been shown in previous reports (1) from this laboratory that, in the normal dog, when the spleen is removed little or no change in the elimination of iron occurs. On the other hand, in man, splenectomy, in the course of congenital hemolytic jaundice (2) and pernicious anemia (3), is followed by a decrease in the elimination of iron equal to about 40 per cent. Moreover, in hemolytic jaundice (2) the amount of iron eliminated before splenectomy is twice as great as that taken in the food. These observations have been interpreted as indicating that in diseases of the spleen, accompanied by anemia, splenectomy removes or inhibits some factor responsible for blood destruction. This view is supported by the observation that after splenectomy there occurs also a decrease in the elimination of urobilin. On the other hand, if one compares the figures for iron elimination in anemia with those of normal individuals (4), it is found that the former fall, as a rule, within the range of the latter. This naturally brings up the question of the factors determining the storage and elimination of iron in conditions of blood destruction. Very few data concerning this subject are at hand (Table I). The only detailed observations aside from those made in this laboratory are the studies of McKelvy and Rosenbloom (5) in congenital hemolytic jaundice, and of Bayer (6), and of Roth (7) after splenectomy for Banti's disease and hemolytic anemia, respectively.

It is obviously difficult to carry out in man prolonged studies, in the different stages of anemia, of the distribution of iron in the various organs. Only end-results, as in the study of bronzed diabetes of Muir and Dunn (8), are possible. We have, therefore, in the present work attempted, by experiments on animals, to throw some light not only upon the distribution and storage of iron but also upon its elimination in various types of blood destruction due to a single or transient injury. This work we consider to be necessary as a

TABLE I.

Elimination of Iron in Healthy and Anemic Individuals.

Observer.	Sex.	Age.	Iron.		Remarks.
			Intake per day.	Output per day.	
		yrs.	*mg.*	*mg.*	
Lehmann, Mueller, Munk, Senator, and Zuntz (4).	Male.	26	Fasting.	7.3*	Professional fasters; 10 and 6 day periods respectively.
	"	21	"	7.7	
Stockman and Greig (4).	Male.	20	6.2†	6.32‡	Healthy individuals.
			5.6	11.46	
	"	35	6.2	8.33	
	Female.	23	3.5	3.73	
Von Wendt (4).	Male (1st period).		11.0†	9.0‡	Nine periods of observation on two healthy individuals.
	(2nd ").		6.0	11.0	
	(3rd ").		10.0	14.0	
	(4th ").		8.0	9.0	
	(5th ").		17.0	42.0	
	(6th ").		7.0	15.0	
	(7th ").		19.0	24.0	
	(8th ").		28.0	34.0	
	(9th ").		27.0	32.0	
Sherman (4).	Male.		5.7§	5.5‡	Three healthy individuals.
	"		6.5	8.7	
	"		7.1	12.6	
McKelvy and Rosenbloom (5).	Female.	11	8.8†	32.51‡	Congenital hemolytic jaundice; 5 day period.
Roth (7).	Male.	26	90.0§	6.25‡	Hemolytic anemia; splenectomized 3 yrs. previously.
			150.0	4.32	
	"	37	90.0	12.18	Splenectomized 1 mo. previously for trauma of spleen.
			200.0	33.07	
Bayer (6).	Male.	16	240.0†	19.38*	2 wks. after splenectomy for traumatic spleen rupture.
			140.0	7.41	
			130.0	14.54	3 mos. later.
			80.0	5.92	
			300.0	26.73	

* Feces only.

† Iron intake determined by actual analysis.

‡ Urine and feces.

§ Iron intake estimated from tables.

TABLE I—*Concluded.*

Observer.	Sex.	Age.	Iron.		Remarks.
			Intake per day.	Output per day.	
		yrs.	*mg.*	*mg.*	
Bayer (6).	Male.	16	240.0	8.40	Control; fracture of tibia.
			140.0	7.29	
	"	16	130.0	8.57	Control; osteomyelitis operation 14 days previous.
			80.0	3.57	
			300.0	23.49	
	Female.	19	130.0	13.86	Banti's disease; 2¼ yrs. after splenectomy.
	"	25	130.0	10.20	Banti's disease; ½ yr. after splenectomy.
	"	27	60.0	21.46	Basedow's disease; before thymectomy.
			60.0	32.70	3 wks. after.
			60.0	12.83	6 " "
			60.0	19.00	10 " "
	Male.	22	130.0	3.59	Banti's disease; before splenectomy.
Goldschmidt, Pepper, and Pearce (2).	Male.	5	3.77§	8.29*	Congenital hemolytic jaundice. Before splenectomy (10 day period).
			4.56	4.11	After splenectomy (10 day period).
Pepper and Austin (3).	Male.	40	16.5§	17.0*	Pernicious anemia. Before splenectomy (5 day period).
			16.5	10.0	2 wks. after splenectomy (4 day period).

preliminary to further experimental studies of the spleen in its relation to blood destruction, as also of the mechanism by which splenectomy in man, in the course of anemia, causes a decrease in the elimination of iron.

Our procedure, in brief, has been to determine in normal dogs and in dogs rendered anemic by the administration of various hemolytic agents (sodium oleate, toluylenediamine, and hemolytic immune serum), (*a*) the elimination of iron in the urine and feces, and (*b*) the iron content of the liver and spleen.

Observations of this nature are few in number. Corper's (9) figures for iron in the normal spleen of the dog, calculated on the basis of dry weight, are 0.26 to 0.98 per cent. Samuely (10), working likewise with the dog, gives figures for the spleen and liver, in the anemia produced by pyrodine (acetylphenylhydrazine) as 0.3921 gm. and 0.2298 gm. respectively per 100 parts of dry weight of blood- and fat-free organ and 0.4819 gm. and 0.3299 gm. per 100 parts of dry weight of fat-free but blood-containing organ. In two other animals after recovery from anemia the comparable figures were 0.1892 and 0.0721 and 0.3892 and 0.3307 gm. Tartakowky (11), after feeding iron to dogs with experimental anemia, found that the iron content of the liver rose from 0.1048 to 2.068 gm. and that of the spleen from 0.09 to 0.172 gm. Boycott and Price-Jones (12), in a study of experimental trypanosome anemia in rabbits, found the iron of the liver to be double that of normal animals, while the iron of the spleen was increased twenty times. As to the elimination of iron in normal animals, von Voit's (13) fasting dog put out 0.6 mg. per day per kilo of body weight, while Gottlieb's (14) dog on an iron-poor diet eliminated 0.34 mg. Hamburger's (15) figures with animals on a meat diet show that the output of iron in the urine is increased but slightly when large amounts of iron sulfate are fed. No other figures for the dog appear to be available.

An interesting series of experiments on rabbits, analogous to ours on the dog, has been made by Muir and Dunn (16). They produced rapid, severe anemia by the use of an hemolytic immune serum, and when the anemia was sufficiently severe, as shown by daily blood counts, the liver, spleen, kidney, stomach, and intestine were removed, after the circulating blood had been washed out, and the iron content was determined and compared with that of normal organs similarly treated. At the same time the blood volume was estimated by bleeding and a colorimetric determination of the hemoglobin content of the washings. By comparing, therefore, the iron content of normal and anemic organs, the excess in the latter was easily estimated, and by comparing hemoglobin figures during life with total blood volume at death, the amount of iron lost from the blood could be determined. Their conclusions are as follows: (1) With destruction of more than half the blood within 3 days, nearly all the iron from injured cells is deposited in the liver, spleen, and kidneys; a certain amount escapes in the urine when hemoglobinuria is present and the amount deposited in the kidneys is roughly proportional to the hemoglobinuria. (2) A third of the total iron of the blood may be deposited in the liver, spleen, and kidneys in 24 hours. Their figures show that in anemia the spleen contained three times, and the liver five times the amount of iron found in normal animals.

In a second series (17) the organs were examined when blood counts showed that the anemia had been completely repaired. The iron content of the organs was found to be only slightly above that of normal organs, indicating that the iron deposited as the result of hemolysis had been nearly all absorbed during the process of blood regeneration—presumably utilized, according to Muir and

Dunn, in the formation of new blood cells. In neither of the investigations was the elimination of iron in urine and feces determined.

Methods.

As our principal object was to determine the relation between the amount of iron lost and that stored in certain organs, we limited our study to the estimation of iron in the urine and feces on the one hand, and in the liver and spleen on the other. We have used blood counts to obtain an approximate idea of the degree of blood destruction, but have not attempted to determine the actual total blood destruction. In the earlier experiments, to provide against error dependent upon a possible variation in the iron content of the organs of normal dogs, a portion of the liver and of the spleen was removed at a preliminary operation,[1] and the iron content determined. When later the same animal was used for the study of anemia, exact control figures for the same animal were thus at hand.

The intake of iron in food was not determined in these experiments. Previous studies (1) of iron metabolism in this laboratory have shown that with the diet used, the iron intake is relatively constant. As the diet was the same in both the control and experimental periods we have felt warranted, therefore, in ascribing changes in elimination and storage of iron as due to the experimental lesion produced.

We have also considered it unnecessary to wash out the liver and spleen in order to remove contained blood. The content of blood in each organ is relatively constant, and as it was impossible to remove the blood from the pieces of organs taken out at the preliminary control operations, we have felt that our figures would be more nearly comparable if this was not done at autopsy.

The amount of iron in the urine was determined by Wolter's (18) modification of Neumann's method, and in the feces, liver, and spleen according to Neumann's method (19), with certain modifications. A weighed amount of the substance, calculated to contain from 3 to 5 mg. of iron,[2] is transferred quantitatively to a 750 cc. Kjeldahl

[1] All operations were performed under ether anesthesia.

[2] For feces, weigh out 3 gm.; for spleen, 1.5 gm.; for liver, 2.5 gm.

flask; 10 gm. of potassium sulfate and 30 cc. of concentrated sulfuric acid are then added; the contents of the flask are heated over a free flame and the heating is continued for 15 minutes after the liquid assumes a straw color. After cooling[3] for about 5 minutes, 75 cc. of cold distilled water are slowly added, and after transfer[4] to a 500 cc. Erlenmeyer flask, the mixture is diluted up to about 150 cc. The rest of the analysis is carried out as described by Neumann.[5]

As far as accuracy is concerned, there is little to choose between this modified method of ashing and that of Neumann, for corresponding results are obtained with each. However, considering the factors of time and convenience of manipulation, a marked difference is noted in favor of our modification. With the use of sulfuric acid and potassium sulfate, the ashing is complete in from 1 to 1½ hours with practically no further attention after setting up. On the other hand, Neumann's method, involving as it does the use of nitric acid in addition to sulfuric acid, requires almost constant attention for a long period of time. Another advantage of the modification is that it does away with objectionable nitric acid fumes during ashing, and obviates the necessity of subsequent boiling to remove all traces of nitric acid.

The dogs, kept in metabolism cages, were fed on a standard diet containing 0.4 gm. of nitrogen per kilo and 70 calories per kilo of body weight. The diet consisted of beef heart, lard, bread crumbs, sugar, a little salt, and sufficient bone ash to ensure well formed feces. The food was mixed with about 300 cc. of water, the intake of the latter being constant for each day, except in such animals as received toluylenediamine *per os*. The animals were not catheterized, the urine being collected daily and preserved by cold storage; the feces were marked by carmine.

[3] Too much cooling should not be allowed, otherwise the addition of water will cause solidification, which is not desirable.

[4] In the analysis of feces, it is necessary at this point to filter off the calcium sulfate, care being taken to wash the filter thoroughly with hot water.

[5] Where Neumann suggests the bubbling of air through the solution to prevent overboiling, we have found it practicable to use glass beads. The water which evaporates during the boiling is replaced in order to prevent the potassium sulfate from crystallizing out during the cooling attending the filtration.

The dogs were placed on the standard diet a few days before the beginning of an experiment. After a period of 1 week, during which the normal elimination of iron in the urine and feces was determined, the animals were operated upon[6] and pieces of the liver and spleen were removed for the determination of the normal iron content.[7] Upon recovery, the animals were again placed under observation and the urine and feces analyzed to determine whether there had been any change in the iron output. Following this, an hemolytic agent was administered, and after studying the output of iron for varying periods of time (3 to 10 days), the animals were chloroformed, and the liver and spleen taken for analysis. In all cases these organs, together with the contained blood, were dried on the water bath, ground up into a fine powder, dried again at 100°C., and analyzed for iron. The feces were analyzed in a similar manner. Throughout the work all precautions were taken to prevent the introduction of extraneous iron.

Anemia was produced in one of three ways: (*a*) sodium oleate (Merck) given intravenously, (*b*) toluylenediamine (Merck) administered *per os*, and (*c*) hemolytic immune serum[8] injected intravenously.

Hemoglobin estimations and red blood cell counts were made from time to time to determine the course and severity of the anemia produced.

Influence of Sodium Oleate.

Sodium oleate was selected as an hemolytic agent which produces a single injury to the red cells without continued action. The hemoglobin is set free in the circulating blood and is rapidly removed in large part by the kidney without the retention in the organs of agglutinated and injured red cells gradually undergoing disintegration.

[6] For the operative work we are indebted to Dr. J. E. Sweet of the Department of Surgical Research.

[7] As the results obtained for normal animals were so uniform, this procedure was abandoned in some of the later experiments.

[8] The hemolytic immune serum was prepared by injecting a rabbit repeatedly with 5 cc. of dog blood at intervals of about 5 days. 1 week after the fifth injection, the rabbit was bled and the serum collected.

The results of two of our experiments with this substance are shown in Tables II and III.

In a third experiment similar results were obtained. There was an increase of iron in the urine, corresponding to the period of hemoglobinuria, with a return to normal figures when the urine cleared. No change in the percentage of iron in the feces or liver and spleen was evident. These results indicate that in hemolysis caused by a

TABLE II.

Dog 1. Effect of Sodium Oleate.

Period.	Iron* in.					Remarks.
	Urine per day.	Feces per day.		Spleen.	Liver.	
	mg.	*mg.*	*per cent*	*per cent*	*per cent*	
Oct. 1–7. Control period (7 days).	1.2	19.4	0.138			
Oct. 8. Control operation.				0.187	0.069	Removal of small portions of organs.
Oct. 11–17. Control period (7 days).	1.9	20.7	0.100			
Oct. 23. Sodium oleate given.						100 cc. of 1 per cent solution in vein.
Oct. 24.	8.0					Hemoglobinuria.
" 25, 26.	2.0					No hemoglobinuria.
" 26. Sodium oleate given.		14.6 (Oct. 23–29)	0.100			200 cc. of 1 per cent solution in vein.
Oct. 27, 28.	9.5					Hemoglobinuria.
" 29, 30.	2.0					No hemoglobinuria.
Nov. 1.				0.173	0.069	Animal chloroformed.

*In this, as in all the other tables, the percentage of iron is calculated in terms of dry weight.

simple hemolytic agent the free hemoglobin is rapidly removed by the kidney and the increase of iron in the urine represents the iron of this hemoglobin. If hemoglobin does not appear in the urine the iron of the urine is not increased in amount. Analyses of the organs indicate that if hemoglobin is retained, the amount is insufficient

to change the iron content of the spleen or liver. No change is seen in the figures for the feces.

TABLE III.

Dog 2. Effect of Sodium Oleate.

Period.	Iron in.					Remarks.
	Urine per day.	Feces per day.		Spleen.	Liver.	
	mg.	*mg.*	*per cent*	*per cent*	*per cent*	
Oct. 9–15. Control period (7 days).	1.3	20.1	0.117			
Oct. 19. Control operation.				0.175	0.112	Removal of small portions of organs.
Oct. 27. Sodium oleate given.						Hemoglobin 90 per cent; red blood cells, 5,260,000. 200 cc. of 1 per cent solution in vein.
Oct. 27–29.	1.2	22.3 (Oct. 27–31)	0.114			Oct. 28. Hemoglobin 85 per cent; red blood cells 5,180,000. No hemoglobinuria.
Oct. 30. Sodium oleate given.	1.4					300 cc. of 1 per cent solution in vein. No hemoglobinuria.
Oct. 31.	3.9			0.181	0.108	Hemoglobin 72 per cent; red blood cells 4,950,000. Slight hemoglobinuria. Animal chloroformed.

Influence of Toluylenediamine.

In the second series of experiments toluylenediamine was selected because it produces a destruction of blood characterized, when moderate doses are given, by choluria rather than by hemoglobinuria, thus making it possible to determine the elimination of iron in a type of anemia in which, if iron is excreted by the kidney, the excretion is not complicated by the presence of free hemoglobin. Moreover, toluylenediamine, unlike sodium oleate, which produces its

effect in a short space of time, has a more prolonged cumulative effect and destroys red cells, according to Joannovics and Pick (20) and Maidorn (21), in part at least, through an hemolytic agent which is intimately connected with the products of fatty degeneration. The mechanism of blood destruction is, therefore, of a more complicated type than that due to sodium oleate. An experiment of this type, with intoxication for 1 week only, is shown in Table IV.

TABLE IV.

Dog 3. Effect of Toluylenediamine.

Period.	Iron in.					Remarks.
	Urine per day.	Feces per day.		Spleen.	Liver.	
	mg.	*mg.*	*per cent*	*per cent*	*per cent*	
Nov. 25–Dec. 1. Control period (7 days).	1.2	19.7	0.101			
Dec. 8. Control operation.				0.187	0.104	Removal of small portions of organs.
Dec. 11–17. Toluylenediamine for 7 days.	1.6	21.6	0.108			0.2 gm. of toluylenediamine *per os* daily. Choluria daily; no hemoglobinuria.
Dec. 18.				0.283	0.146	Animal chloroformed.

In this animal, despite the fact that a moderately severe anemia was present, as shown by 3,300,000 red cells and 58 per cent of hemoglobin on December 17, practically no change in the elimination of iron occurred. On the other hand, the figures show a considerable increase in the percentage of iron in the liver and spleen.

In a second experiment in every way similar, except that toluylenediamine was administered for 9 days instead of 7, analogous results were obtained. The iron in the urine in the period of anemia was 1.6 mg. daily as compared with 1.4 mg. for the control period, and the percentage of iron in the feces, 0.110 and 0.103 for the respective periods. The iron in the spleen and liver, respectively, in the anemia period amounted to 0.299 and 0.154 per cent, as compared with 0.190 and 0.104, respectively, for the normal. The urine

of this animal was constantly bile-stained and the hemoglobin content of the blood fell from 86 to 58 per cent.

In another pair of experiments, toluylenediamine was given for 1 week and discontinued for the same period. The object of these experiments was to determine whether a delayed elimination of iron occurred simultaneously with a resorption of iron from the liver and spleen.

In these and some of the later experiments the preliminary control operation, for the purpose of obtaining tissue for iron analysis, was not done. As may be seen in Table V, the figures for iron in tissues obtained by this preliminary procedure are fairly uniform, averaging 0.104 per cent for the liver and 0.185 for the spleen. In view of this uniformity, the control operation was, therefore, not always done as a necessary part of each experiment, and it was assumed that the normal iron content of the liver is never more than 0.112 per cent, and that of the spleen never more than 0.190 per cent. Reference to Table V will show that these figures are secured by excluding only one extremely low figure, that for the liver of Dog 6.

TABLE V.

Per Cent of Iron in the Liver and Spleen of the Normal Dog.

Dog No.	Liver.	Spleen.
4	0.104	0.187
5	0.104	0.190
6	0.069 (?)	0.187
7	0.098	0.180
8	0.108	0.187
9	0.100	0.188
10	0.112	0.175
Average.....	0.104	0.185

The results of an experiment in which a recovery period of 1 week was allowed after the administration of toluylenediamine are shown in Table VI.

In a second experiment carried out on exactly the same lines, the urine figures in mg. for the three periods were 1.4, 1.6, and 1.5; those for the feces were 18.6, 20.7, and 19.3 with percentages of 0.107, 0.110, and 0.100. The percentage of iron in the liver was 0.132 and in the spleen 0.233. The urine was bile-stained throughout the

period of intoxication. The blood examination gave 101 per cent of hemoglobin in the first period, 70 at the end of the second, and 98 at the end of the third.

These experiments show no evidence of immediate or delayed iron elimination by the kidney or intestine and the figures for iron in the liver and spleen, definitely higher than those for normal organs, would appear to indicate that the increased amount retained, as the result of blood destruction, is not readily given up by these organs.

TABLE VI.

Dog 11. Toluylenediamine with an Interval for Recovery.

Period.	Iron in.					Remarks.
	Urine per day.	Feces per day.		Spleen.	Liver.	
	mg.	*mg.*	*per cent*	*per cent*	*per cent*	
Jan. 19–25. Control period (7 days).	1.5	16.6	0.106			Jan. 26. Hemoglobin 102 per cent; red blood cells 5,240,000.
Jan. 26–Feb. 1. Toluylenediamine for 7 days.	1.4	18.2	0.100			0.2 gm. of toluylenediamine daily. Urine constantly bile-stained. Feb. 1. Hemoglobin 70 per cent; red blood cells 3,580,000.
Feb. 2–8. Recovery period.	1.7	17.2	0.103			
Feb. 9.				0.253	0.140	Hemoglobin 95 per cent; red blood cells 4,990,000. Animal chloroformed.

Influence of Hemolytic Immune Serum.

Hemolytic serum was used in part on account of its definite hemolytic effect, but chiefly because of its agglutinative effect on red cells and their consequent accumulation in the liver, spleen, and lymph nodes. The red cells responsible for the spodogenous tumor of the spleen and enclosed in the endothelial cells of the liver and lymph nodes represent an accumulation of hemoglobin gradually disintegrating and therefore altering profoundly the iron content of these organs. All injections of serum[8] were made intravenously.

Two experiments (Table VII) support the histological evidence that agglutinated and altered red cells are held in the organs immediately after the administration of an agglutinative and hemolytic

TABLE VII.

Effect of Hemolytic Serum.

Dog No.	Period.	Iron in.		Remarks.
		Liver.	Spleen.	
		per cent	*per cent*	
12	Nov. 3. Control operation.	0.098	0.180	
	" 9. Serum given.	0.177	0.287	0.5 cc. of serum per kilo. Died in 3 hrs.
13	Nov. 9. Control operation.	0.100	0.188	
	" 11. Serum given.	0.176	0.301	0.4 cc. of serum per kilo. Died in 3 hrs.

TABLE VIII.

Dog 14. Effect of Hemolytic Serum.

Period.	Iron in.					Remarks.
	Urine per day.	Feces per day.		Liver.	Spleen.	
	mg.	*mg.*	*per cent*	*per cent*	*per cent*	
Dec. 6–12. Control period (7 days).	1.7	11.0	0.110			
Dec. 15. Control operation.				0.108	0.187	Hemoglobin 105 per cent; red blood cells 9,220,000.
Dec. 17–23. Period of anemia* with 4 days of hemoglobinuria.	6.1	31.2	0.120			Dec. 22. Hemoglobin 55 per cent; red blood cells 5,630,000.
Dec. 24.				0.204	0.447	Animal chloroformed.

*On Dec. 17, the animal received 0.06 cc. of serum per kilo and on Dec. 18, 0.09 cc. per kilo. These injections were followed by the appearance of bile pigment in the urine. On Dec. 19, 0.18 cc. of serum per kilo was administered; this was followed by hemoglobinuria persisting for 4 days.

serum. The dogs in question died within 3 hours after receiving the serum, and the increase in iron content cannot therefore be ex-

plained by a deposition of free iron but as due to the masses of erythrocytes held in the endothelial cells and small vessels of the organs. It should be noted that in these two experiments we returned to the practice of performing a preliminary control operation in order to determine the exact amount of iron in the normal organs. The estimation of iron in the urine and feces was impossible on account of the short time the animals survived the injection.

TABLE IX.

Dog 15. Effect of Hemolytic Serum.

Period.	Iron in.					Remarks.
	Urine per day.	Feces per day.		Liver.	Spleen.	
	mg.	*mg.*	*per cent*	*per cent*	*per cent*	
Control period (7 days).	1.8	21.1	0.098			Mar. 28. Hemoglobin 98 per cent; red blood cells 5,120,000.
Mar. 29–Apr. 4. Period of anemia* with 3 days of hemoglobinuria.	11.1	30.8	0.105			Mar. 30. Hemoglobin 50 per cent; red blood cells 3,980,000.
Apr. 5–11. Choluria but no hemoglobinuria.	1.5	23.6	0.103			Apr. 10. Hemoglobin 40 per cent.
Apr. 17.				0.245	0.490	Hemoglobin 30 per cent; red blood cells 2,780,000. Animal chloroformed.

*On Mar. 29, the injection of 0.17 cc. of serum per kilo caused the appearance of bile pigment in the urine; 0.23 cc. on Mar. 31 caused hemoglobinuria lasting 3 days, and the persistence of bile pigment until the end of the experiment.

In two other experiments of the same type, with smaller doses of serum (0.25 cc. per kilo) and without preliminary operation, the animals were killed by chloroform at the end of 18 and 24 hours respectively. The same increase of iron in the liver and spleen was found—0.176 and 0.178 per cent for the respective livers and 0.318 and 0.307 per cent for the spleens.

In a fifth experiment (Table VIII), with preliminary operation,

the analysis of the feces shows that although the total iron in the feces is increased, the percentage remains practically unchanged. The increase of iron in the urine is due to the presence of hemoglobin. As usual, the iron content of the liver and spleen is increased.

In the final experiment (Table IX) of the series, the observations were continued for 2 weeks through a period of prolonged severe anemia. This experiment shows clearly that the iron of the urine is increased only in the presence of hemoglobinuria. During the period of April 5 to 11, blood disintegration continued, as shown by the continued fall of hemoglobin and red cell count and by the presence of bile in the urine, but the iron content of the urine was lower than in the control period. The total elimination of iron in the feces was appreciably higher in the first period of anemia, but the percentage was altered but little. These variations are to be explained, as also those given in Table VIII, by differences in the bulk of feces. The figures for iron in the liver and spleen showed the usual increase.

SUMMARY.

Blood destruction due to a single injury, as by sodium oleate, or acting through a short period of time, as by toluylenediamine or hemolytic immune serum, is not characterized, in the absence of hemoglobinuria, by an increased elimination of iron in the urine. This holds, not only for the evanescent injury caused by sodium oleate, but also for the severe type caused by hemolytic immune serum, in which a progressive destruction of the blood may persist for 2 weeks or more with constant evidence of the disintegration of erythrocytes as shown by bile pigment in the urine. This finding is in accord with previous investigations of anemia in both man and animals.

Likewise, no striking increase is evident, under such circumstances, in the percentage of iron excreted in the feces. The total amount of iron in the feces has been notably increased in two experiments with hemolytic serum, but as the percentage was not appreciably altered, the difference depends presumably on variations in the bulk of feces rather than upon increased elimination.

This evidence of the power of the body to conserve the iron re-

sulting from erythrocytic disintegration is further emphasized by the increased storage of iron in the liver and spleen. This storage is not evident in the slight transient injury caused by sodium oleate, but in the more prolonged anemia due to toluylenediamine and hemolytic serum, the iron content of the spleen and liver is greatly increased, and in the case of anemia due to hemolytic serum is more than doubled.

In view of this definite evidence of the power of the animal body to conserve iron, it is obvious that in the hemolytic anemias in man characterized by excessive elimination of iron in the feces, some other factor than mere blood destruction is operative. Theoretically, it may be assumed that it is a disturbance of the mechanism concerned in the retention or conservation of iron. This phase of the problem we are now studying in experimental types of chronic progressive anemia due to a persisting and ever active cause; and we trust that the studies may throw some light on the iron exchange in anemia and in particular upon the class of hemolytic anemias so markedly benefited by splenectomy.

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A STUDY OF THE ACIDOSIS, BLOOD UREA, AND PLASMA CHLORIDES IN URANIUM NEPHRITIS IN THE DOG, AND OF THE PROTECTIVE ACTION OF SODIUM BICARBONATE.

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PLATES 57 AND 58.

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HISTORICAL.

Numerous investigations have been made concerning the relation between nephritis and the alkalinity of the blood. The earliest studies were made by titration of the blood serum or of a dialysate of the serum with a weak acid, usually phosphoric or tartaric acid with litmus paper as an indicator. By such a method von Jaksch (1) noted a decrease in the alkalinity of the blood in uremia and concluded that the diminished alkalinity of the blood is a factor in inducing some of the phenomena of uremic intoxication. Brandenburg (2, 3), using a similar method, confirmed von Jaksch's finding. The methods employed by these investigators are, however, admittedly inadequate. In 1912 Straub and Schlayer (4), using Haldane's method, found a diminution in the carbon dioxide content of the alveolar air in uremia and thus established on a secure basis the association of acidosis with uremia. Von Noorden (5), however, considered that although a slight grade of acidosis may be present in uremia, it represents merely an associated phenomenon (perhaps the result of inanition) and not the underlying cause of the intoxication. Sellards (6) observed that a larger quantity of sodium bicarbonate must be ingested by certain nephritics, in order to render their urine alkaline, than is the case with normal individuals. This phenomenon he called "increased tolerance to sodium bicarbonate" and concluded that it indicates a condition of acidosis. In 1913 Palmer and Henderson (7), applying the principle of Sellards, but with a more highly developed method for determining the acidity of the urine, found evidence of some degree of acidosis in many pathological conditions. Among these were certain acute infections, severe anemias, the cachexias of malignant neoplasms, and certain types of nephritis.

There are also numerous investigations concerning the effect of alkali and acid upon the alkalinity of the blood and of the urine. Miquel (8) noted a diminution of the alkalinity of the blood of normal animals following the administration

of acids. Gaethgens (9), on the other hand, observed a rapid excretion of the acid introduced without detecting any depletion of the bases of the blood. Lassar (10) using the tartaric acid titration method with litmus paper as an indicator reported a slight, but unquestionable decrease in the alkalinity of the blood of a dog to which acid had been administered. A more reliable method was that employed by Walter (11) in 1877, who made use of the fact that the carbon dioxide content of the blood is proportional to the content of available base in the blood. He demonstrated that when an animal is treated by injecting an acid into the blood stream, the blood undergoes a diminution in its content of base as shown by its diminished carbon dioxide content. He also showed that under such conditions there is an increased ammonia excretion in the urine and eventually a respiratory death, which may be delayed by the administration of soda. Von Hösslin (12) found an intimate relation between the acidity of the urine as determined by the method of Moritz and the amount of albumin and casts in the urine. He observed that following a lowered acidity of the urine after administration of soda, the albumin in the urine as well as casts diminishes, and, furthermore, that such lowered acidity is accompanied by an improvement in the renal functional capacity as indicated by a better excretion of sodium chloride. Von Hösslin (13), therefore, advocated the administration of sodium bicarbonate in order to reduce the acidity of the urine in nephritis. He pointed out that the initial acidity of the urine is no gauge of the amount of soda that must be given to render the urine alkaline. Henderson and Palmer (14) have investigated the effect of acid ingestion upon the hydrogen ion concentration in urine and found a constant increase of acidity in the urine after the ingestion of considerable amounts of acid, but they were not able to produce a urine of an acidity as great as that common in many pathological conditions. Scheltema (15) also obtained favorable effects from the administration of alkali to nephritic individuals. Henderson and Palmer (16), in a study of the factors of acid excretion in nephritis, found "first, that the urinary concentration of ionized hydrogen is, in a statistical sense, increased in the various forms of nephritis; and secondly that such pathological states are frequently marked by a condition of acidosis." They found (17) a renal retention of alkali in those cases of nephritis in which excretion of ammonia is diminished in the urine.

EXPERIMENTAL.

The present investigation was undertaken to study the development of acidosis in nephritis produced by uranium nitrate and the relation of this acidosis to the changes in urea and chlorides of the blood, and also to study the effect of administration of sodium bicarbonate upon all these factors. In these experiments the following determinations were made: (1) the carbon dioxide content of

the plasma and the hydrogen ion concentration of the serum, (2) the urea nitrogen of the blood, (3) the chlorides of the plasma, and (4) the reaction of the urine and its content of albumin and casts. For the carbon dioxide content of the plasma, the Van Slyke-Stillman-Cullen (18) method was used; for the hydrogen ion concentration of the serum, Marriott's modification of the Levy-Rowntree-Marriott method (19, 20); for urea determinations, the Van Slyke-Cullen method (21, 22); and for the plasma chlorides, the method of McLean and Van Slyke (23). In every instance duplicate determinations were done.

The blood for these determinations, except for the hydrogen ion concentration of the serum, was obtained by drawing the blood from the external jugular vein through a tube passing to the bottom of a centrifuge tube containing either sodium oxalate or potassium oxalate crystals and a layer of paraffin oil which, floating on the surface of the blood, excluded contact with the air. The amount of oxalate employed was about 1 per cent by weight of the amount of blood. A portion of the whole blood was removed for urea determination and the remainder was centrifuged. Of the plasma so obtained, 1 cc. was transferred directly from beneath the paraffin oil to the Van Slyke burette for determination of its carbon dioxide content. The results of these determinations are given in the tables in the columns headed "direct." The remainder of the plasma was removed from the cells and used for chloride determination and for the carbon dioxide content after saturation in an atmosphere of 5.5 per cent carbon dioxide at room temperature. This saturation was performed by introducing about 3 cc. of the plasma into a 250 cc. separatory funnel and filling this with normal alveolar air by exhaling deeply five times through the funnel. The funnel was then closed and the plasma shaken in this atmosphere for 1 minute. 1 cc. portions were then transferred to the burette for analysis. The results of these determinations are given in the columns headed "funnel." These readings were always completed within an hour of the drawing of the blood. For the hydrogen ion concentration of the serum, blood was drawn directly from the vein into a small test-tube and the serum separated by centrifuging. In addition, plasma was examined by Marriott's method. The columns headed

"direct" represent the reading obtained at the close of dialysis; the columns headed "A. B." are the readings after the removal of carbon dioxide from the dialysate by aeration.

Plasma Carbon Dioxide Content, Blood Urea, and Plasma Chlorides in Dogs with Uranium Nephritis.

Since it has been noted by MacNider (24) that the age of a dog has an important bearing on the degree of nephritis caused by uranium nitrate, dogs of the same age have been used as far as possible. The importance of diet in determining the toxicity of uranium nitrate has been shown by Opie (25). Animals have been shown to be more susceptible to this poison when kept upon a diet rich in meat than when upon a diet rich in carbohydrates. For this reason, the animals employed have received a constant diet of milk and dog biscuit with no meat. The animals were fed daily at 5 p.m. In regard to the method of injection, the crystalline uranium nitrate has been dissolved in distilled water and given to the dogs, at the first injection in the proportion of 0.015 gm. per 10 kilos of body weight. After an interval of 20 days another injection of the same dose was given, followed by two subsequent injections at 10 day intervals. The amount of uranium given was increased in the third and fourth injections; in the third injection 0.0185 gm. and in the fourth injection 0.045 gm. per 10 kilos of body weight. The object of the increased doses was to produce severe nephritis, in which the carbon dioxide content, urea, and chlorides of the blood might undergo a more marked alteration. Finally, at the end of the experiment, the animals were killed and the kidneys examined. Four dogs were used in the experiments. They were bled at 9 a.m. on the days when the blood was studied. Three preliminary examinations were made on each dog before administering either uranium or soda. The results of these examinations are shown in Table I. The results following the administration of uranium to Dogs 1 and 2 are shown in Tables II and III and Text-figs. 1, 2, and 3.

In Dog 1, after the first injection of uranium, the carbon dioxide content of the plasma underwent a considerable diminution in the course of a week, and, at the same time, the urea and chlorides of

TABLE I.

Normal Dogs.

	Van Slyke method.		Marriott method.				Urea nitrogen in 100 cc. of blood.	Chlorides in 1 cc. of plasma.	Urine.		
			Serum.		Plasma.						
	Direct.	Funnel.	Direct.	A. B.	Direct.	A. B.			Reaction.	Albumin	Casts.
	per cent	*per cent*	*log.*	*log.*	*log.*	*log.*	*mg.*	*mg.*			
Dog 1	55	58	7.5	8.0	7.4	7.9	11	5.6	Acid.	—	—
	63	66	7.9	8.1	7.5	7.8	13	5.9	"	—	—
	62	65	7.8	8.0	7.4	7.9	12	6.0	"	—	—
Average.....	60	63	7.7	8.0	7.4	7.9	12	5.8			
Dog 2	56	61	7.4	7.9	7.3	7.7	14	5.4	Acid.	—	—
	60	65	7.8	8.0	7.4	7.7	13	5.9	"	—	—
	61	65	7.6	8.0	7.4	7.8	12	5.6	"	—	—
Average.....	59	64	7.6	8.0	7.4	7.7	13	5.6			
Dog 3	55	62	7.8	7.9	7.6	7.9	13	5.8	Acid.	—	—
	56	62	7.9	8.0	7.6	8.0	13	5.9	"	—	—
	57	64	7.9	8.0	7.4	8.0	13	5.8	"	—	—
Average.....	56	63	7.9	8.0	7.6	8.0	13	5.8			
Dog 4	55	60	7.8	7.9	7.3	7.9	13	5.5	Acid.	—	—
	58	65	7.8	7.9	7.4	7.7	15	6.0	"	—	—
	60	66	7.9	8.0	7.4	7.8	14	5.8	"	—	—
Average.....	58	64	7.8	7.9	7.4	7.8	14	5.8			

Extent of Variability.

Van Slyke method.		Marriott method.				Urea nitrogen.	Chlorides.
		Serum.		Plasma.			
Direct.	Funnel.	Direct.	A. B.	Direct.	A. B.		
per cent	*per cent*	*log.*	*log.*	*log.*	*log.*	*mg.*	*mg.*
55–63	58–66	7.4–7.9	7.9–8.1	7.3–7.6	7.7–8.0	11–15	5.4–6.0

TABLE II.

Dog 1. Weight 8 kilos.
First injection, 2 p.m., July 3, 1916.

Day.	Date.	Van Slyke method.		Marriott method.				Urea nitrogen in 100 cc. of blood.	Chlorides in 1 cc. of plasma.	Urine.		
				Serum.		Plasma.						
		Direct.	Funnel.	Direct.	A. B.	Direct.	A. B.			Reaction.	Albumin.	Casts.
		per cent	*per cent*	*log.*	*log.*	*log.*	*log.*	*mg.*	*mg.*			
Average		60	63	7.7	8.0	7.4	7.9	12	5.8	Acid.	–	–
1	*1916* July 3	12 mg. of uranium nitrate at 2 p.m.										
2	July 4											
3	" 5	57	63	7.8	7.9	7.1	7.6	21	6.3	Acid.	Tr.	–
4	" 6									"	"	–
5	" 7	53	56	7.7	7.8	7.3	7.7	28	6.7	"	"	+
6	" 8	48	55	7.1	7.8	7.0	7.8	32	6.7	"	++	+
7	" 9									"	+	+
8	" 10	45	50	7.1	7.7	6.9	7.9	36	6.8	"	+	+
9	" 11	40	46	7.0	7.8	6.9	7.8	23	7.0	"	+	+
10	" 12									"	+	+
11	" 13	55	60	7.2	7.9	7.1	7.8	21	6.0	"	+	–
12	" 14									"	+	–
13	" 15	60	65	7.5	7.7	7.3	7.6	19	6.3	"	+	–
14	" 16									"	+	–
15	" 17									"	Tr.	–
16	" 18	58	60	7.7	8.0	7.3	7.8	21	5.8	"	"	–
17	" 19									"	"	–
18	" 20	60	65	7.8	7.9	7.4	7.7	16	5.8	"	"	–
19	" 21									"	"	–
20	" 22	63	65	7.9	8.0	7.4	7.9	16	6.2	"	"	–
21	" 23											
22	July 24	12 mg. of uranium nitrate at 9 a.m.								Acid.	Tr.	–
23	July 25									Acid.	Tr.	–
24	" 26			7.7	7.8	7.3	7.4	23	6.6	"	+	–
25	" 27									"	+	+
26	" 28			7.6	7.8	7.3	7.8	26	6.6	"	++	+
27	" 29									"	++	+
28	" 30									"	++	+
29	" 31			7.5	7.8	7.3	7.8	20	6.3	"	++	+
30	Aug. 1									"	+	+
31	" 2			7.6	7.8	7.4	7.7	21	6.2	"	+	–
32	" 3									"	Tr.	–
33	" 4	60	65	7.7	7.8	7.4	7.6	21	6.1	"	"	–

TABLE II—*Concluded.*

Day.	Date.	Van Slyke method.		Marriott method.				Urea nitrogen in 100 cc. of blood.	Chlorides in 1 cc. of plasma.	Urine.		
				Serum.		Plasma.						
		Direct.	Funnel.	Direct.	A. B.	Direct.	A. B.			Reaction.	Albumin.	Casts.
33	Aug. 4	15 mg. of uranium nitrate at 9 a.m., after bleeding.								Acid.	Tr.	−
		per cent	*per cent*	*log.*	*log.*	*log.*	*log.*	*mg.*	*mg.*			
34	Aug. 5									Acid.	Tr.	−
35	" 6									"	"	−
36	" 7									"	+	−
37	" 8	48	52	7.8	7.8	7.4	7.8	21	6.5	"	+	−
38	" 9									"	+	−
39	" 10	46	50	7.6	7.8	7.2	7.6	22	6.9	"	+	−
40	" 11									"	+	+
41	" 12	49	55	7.9	8.0	7.4	7.8	22	6.7	"	++	+
42	" 13									"	+	−
43	" 14									"	+	−
44	Aug. 15	36 mg. of uranium nitrate at 9 a.m.								Acid.	+	−
45	Aug. 16	49	53	7.6	7.9	7.4	7.8	29	7.0	Acid.	+	+
46	" 17	45	47	7.4	7.8	7.2	7.7	29	7.1	"	++	+
47	" 18	42	46	7.2	7.8	7.1	7.7	26	7.3	"	++	+
48	" 19	40	47	7.2	7.8	7.1	7.6	25	7.3	"	++	+
49	" 20	45	49	7.3	7.8	7.1	7.6	26	7.4	"	++	+
50	" 21									"	+	+
51	" 22	52	56	7.7	7.8	7.2	7.6	25	7.1	"	+	+

the blood exhibited an increase associated with the appearance of albumin and casts in the urine. It will be noted that the minimum of the plasma carbon dioxide content coincides approximately with the maximum of the blood urea and plasma chlorides in time of occurrence. At about this period casts also appeared in the urine. During the 3rd week after the first injection, the plasma carbon dioxide content returned to its normal level, and on the 20th day, the values for plasma chlorides and blood urea nitrogen were 6.2 and 16 respectively. The chlorides, however, had three times been within normal limits between the 11th and 20th days. Urine at

TABLE III.

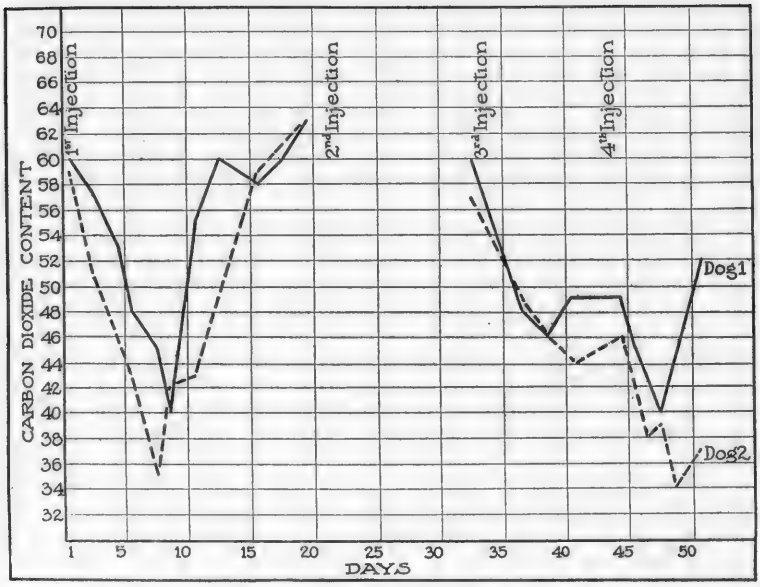
Dog 2. Weight 27 kilos.
First injection, 2 p.m., July 3, 1916.

Day.	Date.	Van Slyke method.		Marriott method.				Urea nitrogen in 100 cc. of blood.	Chlorides in 1 cc. of plasma.	Urine.		
				Serum.		Plasma.						
		Direct.	Funnel.	Direct.	A. B.	Direct.	A. B.			Reaction.	Albumin.	Casts.
		per cent	*per cent*	*log.*	*log.*	*log.*	*log.*	*mg.*	*mg.*			
Average.....		59	64	7.6	8.0	7.4	7.7	13	5.6	Acid.	–	–
1	*1916* July 3	40 mg. of uranium nitrate at 2 p.m.										
2	July 4									Acid.		
3	" 5	51	58	7.8	8.0	7.3	7.8	21	6.4	"	Tr.	–
4	" 6									"	"	–
5	" 7	46	48	7.6	7.8	7.4	7.6	23	6.6	"	+	+
6	" 8	43	47	6.9	7.7	6.8	7.6	25	7.2	"	++	+
7	" 9									"	++	+
8	" 10	35	39	6.9	7.4	6.8	7.3	42	7.3	"	++	+
9	" 11	42	45	7.1	7.8	7.0	7.5	41	6.9	"	++	+
10	" 12									"	++	+
11	" 13	43	46	7.1	7.8	7.0	7.5	42	6.9	"	+	+
12	" 14									"	+	+
13	" 15	49	52	7.5	7.7	7.3	7.5	41	6.6	"	+	+
14	" 16									"	+	–
15	" 17									"	Tr.	–
16	" 18	59	62	7.7	7.9	7.4	7.9	36	6.1	"	"	–
17	" 19									"	"	–
18	" 20	61	63	7.9	8.1	7.3	7.6	26	6.0	"	"	–
19	" 21									"	"	–
20	" 22	63	65	7.8	8.0	7.4	7.8	16	5.9	"	"	–
21	" 23											
22	July 24	40 mg. of uranium nitrate at 9 a.m.								Acid.	Tr.	–
23	July 25									Acid.	Tr.	–
24	" 26			7.8	7.9	7.4	7.8	22	5.9	"	+	–
25	" 27									"	+	+
26	" 28			7.6	7.8	7.3	7.7	26	6.3	"	++	+
27	" 29									"	++	+
28	" 30									"	++	+
29	" 31			7.4	7.7	7.3	7.7	26	6.5	"	++	+
30	Aug. 1									"	+	+
31	" 2			7.5	7.7	7.3	7.6	20	6.1	"	+	?
32	" 3									"	+	?
33	" 4	57	60	7.6	7.7	7.3	7.5	20	6.1	"	+	+

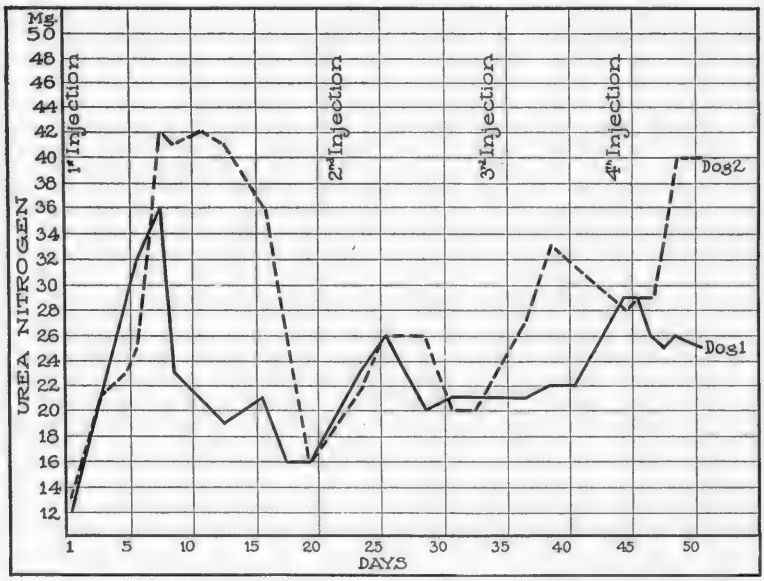
TABLE III—*Concluded.*

Day.	Date.	Van Slyke method.		Marriott method.				Urea nitrogen in 100 cc. of blood.	Chlorides in 1 cc. of plasma.	Urine.		
				Serum.		Plasma.						
		Direct.	Funnel.	Direct.	A. B.	Direct.	A. B.			Reaction.	Albumin.	Casts.
33	Aug. 4	50 gm. of uranium nitrate at 9 a.m., after bleeding.										
		per cent	*per cent*	*log.*	*log.*	*log.*	*log.*	*mg.*	*mg.*			
34	Aug. 5									Acid.	+	+
35	" 6									"	+	+
36	" 7									"	+	+
37	" 8	49	53	7.7	7.7	7.4	7.7	27	6.7	"	++	+
38	" 9									"	++	+
39	" 10	46	48	7.6	7.8	7.3	7.7	33	7.0	"	++	+
40	" 11									"	++	?
41	" 12	44	46	7.7	7.8	7.3	7.8	31	6.9	"	+	+
42	" 13									"	+	+
43	" 14									"	+	+
44	Aug. 15	120 mg. of uranium nitrate at 9 a.m.								Acid.	+	+
45	Aug. 16	46	49	7.6	7.7	7.3	7.6	28	7.0	Acid.	+	+
46	" 17	42	45	7.3	7.7	7.2	7.6	29	7.3	"	+	+
47	" 18	38	40	7.1	7.7	7.0	7.5	29	7.2	"	++	+
48	" 19	39	45	7.1	7.6	7.0	7.5	34	7.3	"	++	+
49	" 20	34	40	7.1	7.7	7.0	7.6	40	7.3	"	++	+
50	" 21									"	++	+
51	" 22	37	43	7.1	7.6	7.1	7.5	40	7.1	"	++	+

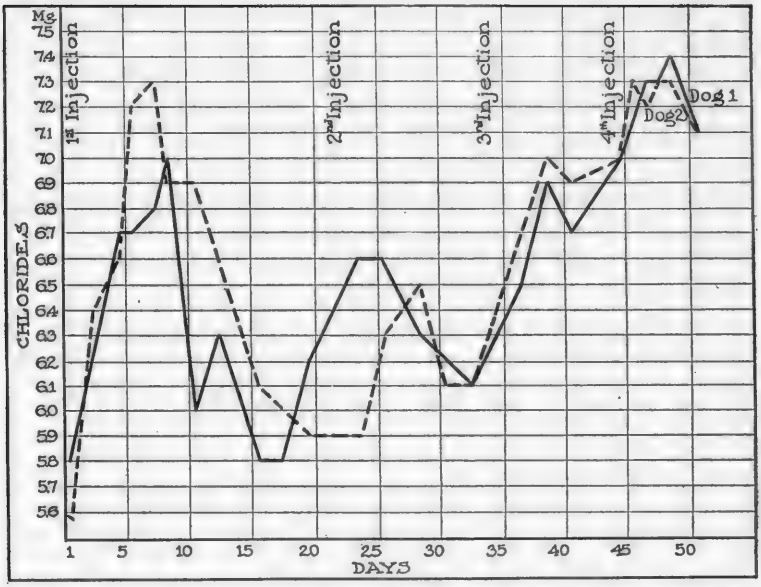
this time still showed the presence of a trace of albumin, although casts were absent. After the second injection of uranium similar changes occurred in the blood urea and plasma chlorides, but less pronounced in degree; the carbon dioxide content could not be studied at this time as the only burette at hand was broken. After the third and larger injection, a marked reduction of the carbon dioxide content occurred and a synchronous marked rise of plasma chlorides; no significant change was noted in the blood urea. It may be noted that casts in the urine appeared rather late. After the fourth injection, which was given before the blood had returned to a normal



TEXT-FIG. 1. Curves showing the carbon dioxide content of the plasma of Dogs 1 and 2 after injections of uranium nitrate. No soda administered.



TEXT-FIG. 2. Curves showing the urea nitrogen in 100 cc. of blood in Dogs 1 and 2 after injections of uranium nitrate. No soda administered.



TEXT-FIG. 3. Curves showing the chlorides in 1 cc. of the plasma in Dogs 1 and 2 after injections of uranium nitrate. No soda administered.

condition, the minimum of plasma carbon dioxide content and the maximum of plasma chloride coincided approximately in their occurrence about the 4th day after injection. During this last period the chlorides in the blood were much higher and the albumin in the urine was much greater in quantity than after the previous injections. The increase in the quantity of blood urea, however, was less than after the first injection.

In the second dog nearly identical relations in the carbon dioxide content, urea, and chlorides of the blood and albumin and casts in the urine were observed as in the first dog. We may, however, note the following slight differences. After their reappearance 3 days after the second injection, the urinary casts persisted throughout the experiment. After each injection the carbon dioxide content, the chloride content, and the blood urea showed greater disturbances than in the first dog. This is prob-

ably to be attributed to the much larger absolute dose of uranium, although the same dose was used per kilo. We may conclude from these experiments (Dogs 1 and 2) that the plasma carbon dioxide content undergoes a considerable diminution in uranium nephritis, while the plasma chlorides and blood urea increase, and, furthermore, that the occurrence of the minimum content of carbon dioxide coincides approximately with the occurrence of the maximum content of chlorides and of urea in the blood. Both the carbon dioxide content and plasma chlorides returned nearly to their normal condition 2 to 3 weeks after the first injection. Urea, on the other hand, did not return quite to its normal level at any period, although 3 weeks after the first injection, the change from the normal value was slight. Albumin after appearing did not disappear entirely from the urine, although casts occasionally did.

Influence of Sodium Bicarbonate on the Plasma Carbon Dioxide Content, Blood Urea, and Plasma Chlorides in Dogs with Uranium Nephritis.

For this experiment Dogs 3 and 4 were used. Each dog received through a stomach tube 1 gm. of sodium bicarbonate dissolved in 10 cc. of water per kilo of body weight at 9 a.m. throughout the entire period of the experiment. On the 3rd day uranium nitrate was injected. The amounts of the uranium per kilo and the intervals between succeeding injections were exactly the same as in the experiment on Dogs 1 and 2. The blood was taken at 9 a.m., before administering the soda. The results are shown in Tables IV and V and Text-figs. 4, 5, and 6. While the dogs were receiving sodium bicarbonate alone, that is, before the giving of uranium, a considerable increase of the plasma carbon dioxide content was observed. Following the first uranium injection this carbon dioxide content showed a decrease and at the same time there occurred an increase in the plasma chlorides and blood urea, as in Dogs 1 and 2.

The relative decrease in the carbon dioxide content following uranium in the dogs receiving soda is comparable with that in the dogs receiving no soda, but the absolute level reached was not so low, because of the higher level already existing when the uranium

TABLE IV.

Dog 3. Weight 13.5 kilos.
First injection, 9 a.m., July 5, 1916.
July 3. 12 a.m. 13.5 gm. of sodium bicarbonate + 135 cc. of water.
From July 4 sodium bicarbonate + water was given every day, after bleeding.

Day.	Date.	Van Slyke method.		Marriott method.				Urea nitrogen in 100 cc. of blood.	Chlorides in 1 cc. of plasma.	Urine.		
				Serum.		Plasma.						
		Direct.	Funnel.	Direct.	A. B.	Direct.	A. B.			Reaction.	Albumin.	Casts.
		per cent	*per cent*	*log.*	*log.*	*log.*	*log.*	*mg.*	*mg.*			
Average		56	63	7.9	8.0	7.6	8.0	13	5.8	Acid.	–	–
	1916											
1	July 3, 2 p.m.	69	71	7.8	8.0	7.5	7.9	13	5.6			
2	" 4									Alkaline.	–	–
3	July 5	20 mg. of uranium nitrate at 9 a.m.								Alkaline.	–	–
4	July 6	66	68	7.8	8.0	7.4	7.9	14	6.1	Alkaline.	Tr.	–
5	" 7	56	60	7.7	8.0	7.3	7.9	15	6.2	"	"	–
6	" 8	55	60	7.4	7.9	6.9	7.8	19	6.2	"	"	–
7	" 9									"	"	–
8	" 10	47	52	7.3	7.7	6.9	7.8	26	6.8	"	"	+
9	" 11									"	"	+
10	" 12	48	50	7.2	7.8	6.9	7.6	29	6.7	"	+	+
11	" 13									"	+	+
12	" 14	55	58	7.4	7.8	7.0	7.6	30	6.0	"	+	+
13	" 15									"	+	–
14	" 16									"	+	–
15	" 17	61	64	7.7	7.9	7.4	7.9	29	6.4	"	Tr.	–
16	" 18									"	"	–
17	" 19	65	68	7.9	8.1	7.4	7.9	32	6.0	"	"	–
18	" 20									"	"	–
19	" 21	70	75	8.0	8.1	7.5	7.9	16	6.0	"	"	–
20	" 22									"	"	–
21	" 23									"	"	–
22	" 24	70	73	7.9	8.1	7.5	7.9	22	5.6	"	"	–
23	" 25											
24	" 26											
25	" 27			8.0	8.1	7.4	7.8	25	5.9	Alkaline.	Tr.	–

TABLE IV—*Concluded.*

Day.	Date.	Van Slyke method.		Marriott method.				Urea nitrogen in 100 cc. of blood.	Chlorides in 1 cc. of plasma.	Urine.		
				Serum.		Plasma.						
		Direct.	Funnel.	Direct.	A. B.	Direct.	A. B.			Reaction.	Albumin.	Casts.
25	July 27	20 mg. of uranium nitrate at 9 a.m., after bleeding.										
		per cent	*per cent*	*log.*	*log.*	*log.*	*log.*	*mg.*	*mg.*			
26	July 28									Alkaline.	+	−
27	" 29			7.8	8.0	7.4	7.9	26	6.5	"	+	−
28	" 30									"	+	−
29	" 31									"	+	−
30	Aug. 1			7.5	7.8	7.3	7.5	17	6.0	"	+	−
31	" 2									"	+	−
32	" 3			7.6	7.8	7.2	7.5	15	5.8	"	+	−
33	" 4									"	+	−
34	" 5	62	55	7.6	7.8	7.3	7.6	15	6.0	"	Tr.	−
35	" 6									"	"	−
36	Aug. 7	25 mg. of uranium nitrate at 9 a.m.								Alkaline.	Tr.	−
37	Aug. 8									Alkaline.	Tr.	−
38	" 9									"	+	−
39	" 10	52	55	7.5	7.8	7.3	7.6	28	6.3	"	+	−
40	" 11									"	+	−
41	" 12	49	52	7.3	7.8	7.2	7.6	23	6.2	"	+	−
42	" 13									"	+	−
43	" 14	50	55	7.5	7.9	7.3	7.5	26	6.4	"	+	−
44	" 15									"	+	−
45	" 16									"	+	−
46	Aug. 17	60 mg. of uranium nitrate at 9 a.m.										
47	Aug. 18	62	67	7.7	7.8	7.4	7.8	28	6.4	Alkaline.	+	−
48	" 19	56	59	7.6	7.8	7.4	7.8	20	6.3	"	+	+
49	" 20									"	+	+
50	" 21	54	61	7.5	7.8	7.3	7.7	19	6.6	"	+	+
51	" 22									"	+	+
52	" 23	58	62	7.5	7.8	7.3	7.6	18	6.3	"	+	+
53	" 24	62	65	7.7	7.9	7.4	7.8	15	6.6	"	+	+

TABLE V.

Dog 4. Weight 19 kilos.

First injection, 9 a.m., July 5, 1916.

July 3. 12 a.m. 19 gm. of sodium bicarbonate + 190 cc. of water.

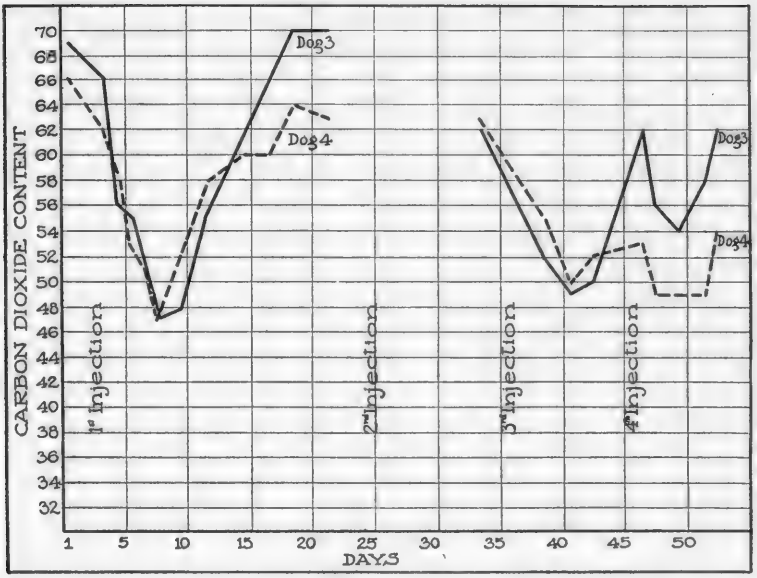
From July 4 sodium bicarbonate + water was given every day, after bleeding.

Day.	Date.	Van Slyke method.		Marriott method.				Urea nitrogen in 100 cc. of blood.	Chlorides in 1 cc. of plasma.	Urine.		
				Serum.		Plasma.						
		Direct.	Funnel.	Direct.	A. B.	Direct.	A. B.			Reaction.	Albumin.	Casts.
		per cent	*per cent*	*log.*	*log.*	*log.*	*log.*	*mg.*	*mg.*			
Average.....		58	64	7.8	7.9	7.4	7.8	14	5.8	Acid.	−	−
	1916											
1	July 3, 2 p.m.	66	70	7.8	8.0	7.5	8.0	13	6.0			
2	" 4									Alkaline.	−	−
3	July 5	29 mg. of uranium nitrate at 9 a.m.								Alkaline.	−	−
4	July 6	62	66	7.9	8.0	7.4	7.8	16	5.6	Alkaline.	Tr.	−
5	" 7	58	60	7.7	7.9	7.3	7.7	18	6.0	"	"	−
6	" 8	53	56	7.4	7.8	7.2	7.7	29	6.3	"	"	−
7	" 9									"	"	−
8	" 10	47	52	7.2	7.6	7.0	7.4	32	6.8	"	+	−
9	" 11									"	+	+
10	" 12	52	55	7.3	7.8	7.2	7.7	32	6.7	"	+	+
11	" 13									"	+	+
12	" 14	58	61	7.4	7.8	7.2	7.6	38	6.3	"	+	+
13	" 15									"	+	−
14	" 16									"	Tr.	−
15	" 17	60	65	7.8	8.0	7.5	7.9	28	6.3	"	"	−
16	" 18									"	"	−
17	" 19	60	66	7.8	7.9	7.4	7.9	36	6.1	"	"	−
18	" 20									"	"	−
19	" 21	64	68	7.9	8.0	7.4	7.8	26	5.8	"	"	−
20	" 22									"	"	−
21	" 23									"	"	−
22	" 24	63	66	7.8	8.0	7.4	7.8	25	5.8	"	"	−
23	" 25									"	"	−
24	" 26									"	"	−
25	" 27			8.0	8.1	7.4	7.7	20	6.0	"	"	−
25	July 27	29 mg. of uranium nitrate at 9 a.m., after bleeding.										
26	July 28									Alkaline.	+	−
27	" 29			7.9	8.0	7.5	7.9	28	6.1	"	+	−
28	" 30									"	+	−
29	" 31									"	+	+

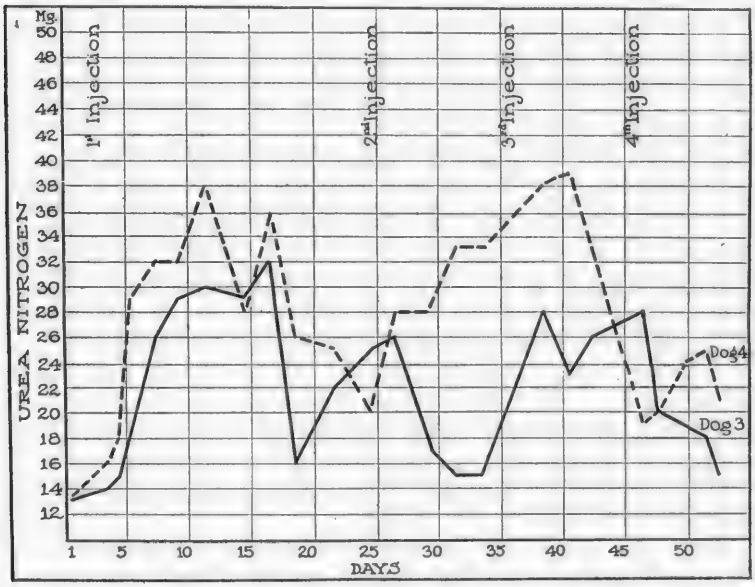
TABLE V—*Concluded.*

Day.	Date.	Van Slyke method.		Marriott method.				Urea nitrogen in 100 cc. of blood.	Chlorides in 1 cc. of plasma.	Urine.		
				Serum.		Plasma.						
		Direct.	Funnel.	Direct.	A. B.	Direct.	A. B.			Reaction.	Albumin.	Casts.
		per cent	*per cent*	*log.*	*log.*	*log.*	*log.*	*mg.*	*mg.*			
30	Aug. 1			7.7	7.9	7.3	7.7	28	5.8	Alkaline.	+	+
31	" 2									"	+	−
32	" 3			7.8	7.9	7.4	7.8	33	6.3	"	+	−
33	" 4									"	+	−
34	" 5	63	65	7.7	7.8	7.4	7.7	33	6.1	"	Tr.	−
35	" 6									"	"	−
36	Aug. 7	36 mg. of uranium nitrate at 9 a.m.								Alkaline.	Tr.	−
37	Aug. 8									Alkaline.	Tr.	−
38	" 9									"	+	−
39	" 10	55	58	7.5	7.6	7.2	7.5	38	5.8	"	+	−
40	" 11									"	+	−
41	" 12	50	52	7.5	7.6	7.3	7.6	39	6.0	"	+	−
42	" 13									"	+	−
43	" 14	52	54	7.6	7.7	7.3	7.5	33	6.4	"	+	−
44	" 15									"	+	−
45	" 16									"	+	−
46	Aug. 17	87 mg. of uranium nitrate at 9 a.m.								Alkaline.	+	−
47	Aug. 18	53	55	7.7	7.8	7.4	7.8	19	6.6	Alkaline.	+	−
48	" 19	49	52	7.6	7.9	7.3	7.7	20	7.0	"	+	+
49	" 20									"	+	+
50	" 21	49	53	7.6	7.7	7.3	7.7	24	6.9	"	+	+
51	" 22									"	+	+
52	" 23	49	52	7.6	7.7	7.3	7.7	25	7.0	"	+	+
53	" 24	54	58	7.7	7.8	7.4	7.7	21	7.0	"	+	+

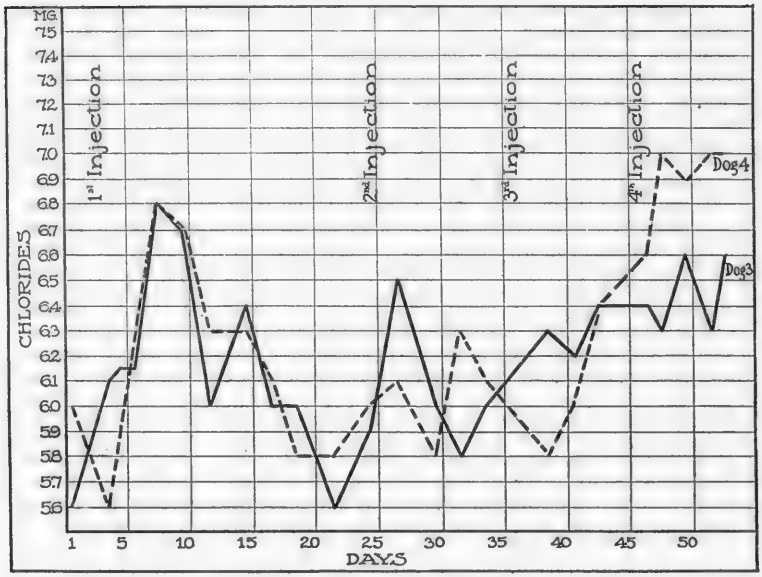
administration was commenced. The increase in the blood urea in the dogs receiving soda is comparable with that in the dogs receiving no soda except following the large fourth injection, when the dogs receiving soda showed a less pronounced rise. The increase in the plasma chlorides was consistently lower throughout the experiments in the dogs receiving the soda. This was especially marked following the fourth injection of uranium. The soda administered was sufficient to keep the urine in these dogs persistently



TEXT-FIG. 4. Curves showing the carbon dioxide content of the plasma of Dogs 3 and 4 after injections of uranium nitrate. Soda administered.



TEXT-FIG. 5. Curves showing the urea nitrogen in 1 cc. of blood in Dogs 3 and 4 after injections of uranium nitrate. Soda administered.



TEXT-FIG. 6. Curves showing the chlorides in 1 cc. of the plasma in Dogs 3 and 4 after injections of uranium nitrate. Soda administered.

alkaline. The albuminuria was persistent from the time of the first appearance, but was definitely less marked than in the animals receiving no soda. Casts were likewise less abundant in these animals and disappeared entirely for longer intervals of time. In general, following the fourth injection, the carbonate dogs showed a considerable difference from the control dogs as to the diminution of carbon dioxide content and as to increase of chlorides and urea in the blood.

The protocols of the histological examination of the kidneys follow.

Dog 1. Weight 8 Kilos. No Soda.

Day of experiment.	Uranium nitrate. Absolute dose.	Relative dose per kilo.
	gm.	*mg.*
1	0.012	1.5
22	0.012	1.5
33	0.015	1.85
44	0.036	4.5
52	Killed.	

Interval before fixation of tissues: a few minutes.

Under the low power the glomeruli appear normal. The epithelium of the proximal convoluted tubules appears swollen, especially in the tubules in the region of the corticomedullary junction. The epithelium of the ascending loops of Henle appear swollen. The medulla appears normal. The medullary rays stand out conspicuously.

Under high power some of the glomeruli exhibit moderate congestion, others appear normal. The epithelium of the proximal convoluted tubules is greatly swollen, but the nuclei are well preserved and only rarely is there any evidence of necrosis. The distal convoluted tubules are almost all normal, except for the presence of serum in the lumina of some. The medulla appears normal.

Diagnosis.—Marked cloudy swelling of the proximal convoluted tubules, especially those near the corticomedullary junction (Fig. 1).

Dog 2. Weight 27 Kilos. No Soda.

	Uranium nitrate.	
Day of experiment.	Absolute dose.	Relative dose per kilo.
	gm.	*mg.*
1	0.040	1.5
22	0.040	1.5
33	0.050	1.85
44	0.120	4.5
52	Killed.	

Interval before fixation of tissues: a few minutes.

Under low power this kidney appears highly abnormal with moderate to marked congestion of the glomerular tufts and with patches of marked epithelial swelling in the proximal convoluted tubules, of interstitial edema, and of serum in the lumina in the cortex, especially in the corticomedullary region. The medulla exhibits marked congestion and edema with some swelling of epithelium and with serum or casts in the lumina.

Under high power congestion of the glomeruli is confirmed. The proximal convoluted tubules show intense epithelial swelling and in many places the nuclei are pale or necrotic. In many places the epithelium has entirely desquamated from these tubules, leaving simply the basement membrane. The distal convoluted tubules and ascending limbs of Henle are in many places encroached upon and compressed by interstitial edema and exhibit degeneration of epithelium and serum or casts in many of the lumina. In some of the epithelial cells of the distal convoluted tubules there is a yellowish pigment resembling hemosiderin.

Diagnosis.—Intense cloudy swelling and necrosis of the epithelium of the proximal convoluted tubules. Moderate cloudy swelling of the epithelium of the ascending limbs of Henle and the distal convoluted tubules. Interstitial edema of the cortex and medulla. Serum and casts in tubule lumina. Congestion of glomerular tufts (Fig. 2).

Dog 3. Weight 13.5 Kilos. Soda.

Day of experiment.	Uranium nitrate. Absolute dose.	Relative dose per kilo.
	gm.	*mg.*
1	0.020	1.5
23	0.020	1.5
34	0.025	1.85
44	0.060	4.5
52	Killed.	

Interval before fixation of tissues: a few minutes.

Under low power the picture is practically normal.

Under high power the glomeruli appear normal. The epithelium of all the tubules is in fair condition with little swelling, although this can be observed in occasional cells in the proximal convoluted tubules. The medullary cells are normal. The lumina are almost entirely free from serum.

Diagnosis.—Approximately normal.

Dog 4. Weight 19 Kilos. Soda.

Day of experiment.	Uranium nitrate. Absolute dose.	Relative dose per kilo.
	gm.	*mg.*
1	0.029	1.5
23	0.029	1.5
34	0.036	1.9
44	0.087	4.6
52	Killed.	

Interval before fixation of tissues: a few minutes.

Under low power the picture is normal except for serum or casts in the lumina of the medullary tubes.

Under high power the glomeruli seem normal. The proximal convoluted tubules show little cloudy swelling. The medulla is normal except for serum or casts in the lumina in a considerable number of the tubules.

Diagnosis.—Slight cloudy swelling. Serum or casts in the medullary tubules (Fig. 3).

It is a well established fact that uranium nitrate produces primarily and most conspicuously degeneration and necrosis of the epithelium of the renal tubules, and especially of the proximal convoluted tubules. The physiological studies of Schlayer and Hedinger (26), Pearce, Hill, and Eisenbrey (27), and others have shown that the reaction of the vascular apparatus of the kidney may be impaired or not by uranium nitrate, depending upon the dosage employed. Christian and O'Hare (28) have found that uranium nitrate causes also a lesion of the glomeruli characterized by the presence of hyaline droplets in the capillary loops and by other changes.

MacNider (29) reported a difference in the degree of pathological change in the kidney following uranium in dogs given sodium carbonate intravenously as compared with control dogs receiving no soda. The most marked difference was seen in the degree of involvement of the epithelium of the convoluted tubules.

It is evident from a study of the histology of the kidneys that the kidneys from both dogs receiving no soda exhibited more pronounced nephritis than those of either of the dogs receiving soda. The most severe nephritis occurred in Dog 2, which received the largest absolute dose of uranium; Dog 1, which received the smallest absolute dose of uranium, but no soda, showed also definitely more marked lesions than did Dogs 3 and 4, which received soda.

From the facts given both in the functional studies and in the pathological examination, it is clear that the nephritis in the dogs receiving sodium bicarbonate is less severe than that in the control dogs.

The toxic effect of uranium for the kidney is usually ascribed to the action of the metal as such. According to the experimental results of MacNider the toxicity of uranium runs parallel with its ability to lead to the formation of various acid bodies, and if the appearance of these substances in the urine is delayed and their amount in the body diminished by the administration of alkali, there is less evidence of the toxic action of the metal. In order to exclude the possibility of the toxic salt of uranium having been itself rendered inert by the direct action of sodium carbonate, he injected two animals with uranium nitrate in which the solvent for the uranium was a 3 per cent solution of sodium carbonate in 0.9 per cent sodium chloride. The toxic effect of uranium was in no way diminished when employed in a 3 per cent solution of the carbonate. He ascribes the protective action of sodium carbonate in uranium nephritis to the neutralization of acid bodies produced by the uranium in the animal economy.

In the study of the hydrogen ion concentration of the blood, the most consistent results were obtained by Marriott's modification of the technique, the figure obtained after aeration of the dialysate from the serum and given in the tables in the column "serum A. B." Determination upon the plasma by the same method gave, on the whole, parallel but probably less consistent results. A comparison of the methods of Marriott and of Van Slyke shows a greater delicacy in the Van Slyke method, so that while the evidences as to the acid

base equilibrium of the blood afforded by the two methods agree, Marriott's method is hardly delicate enough to permit of as satisfactory conclusions in such experiments as the present ones.

Action of Alkali and of Acid upon the Carbon Dioxide Content in Plasma.

Acid was administered by stomach tube to two dogs and alkali to two other dogs. For the acid, hydrochloric acid was chosen, and for the alkali, sodium bicarbonate. 1 cc. of 0.5 per cent hydrochloric acid per kilo of body weight was introduced into the stomach through the stomach tube. When acid was thus administered a diminution of the plasma carbon dioxide content developed, but since in this experiment a severe nephritis resulted, it might be questioned whether the decrease of the carbon dioxide content was produced wholly by the acid administered or in part also by the acidosis of the nephritis.

The microscopic examination gave clear evidence of nephritis in these kidneys.

When alkali (10 per cent sodium bicarbonate) was administered, the plasma carbon dioxide content of the blood showed a constant increase (Tables VI to XI.)

TABLE VI.

Dog 5. Weight 20.5 kilos.
Bled every day at 9 a.m.

Date.	Van Slyke method.		Urine.			Remarks.
	Direct.	Funnel.	Reaction.	Albumin.	Casts.	
1916	*per cent*	*per cent*				
Aug. 5	57	59	Acid.	—	—	
" 6	56	59	"	—	—	Acid at 10 a.m., after bleeding.
" 7	49	52	"	++	++	Died at 11 a.m.

TABLE VII.

Dog 6. Weight 18 kilos.
Bled every day at 9 a.m.

Date.	Van Slyke method.		Urine.			Remarks.
	Direct.	Funnel.	Reaction.	Albumin.	Casts.	
1916	*per cent*	*per cent*				
Aug. 5	58	60	Acid.	—	—	
" 6	55	58	"	—	—	Acid at 10 a.m., after bleeding.
" 7	48	53	"	++	++	

TABLE VIII.

Dog 7. Weight 10.5 kilos.
Bled every day at 9 a.m.

Date.	Van Slyke method.		Urine.			Remarks.
	Direct.	Funnel.	Reaction.	Albumin.	Casts.	
1916	*per cent*	*per cent*				
Aug. 5	56	58	Acid.	—	—	
" 6	55	58	"	—	—	After bleeding, 200 cc. of 10 per cent sodium bicarbonate were given.
" 7	60	63	Alkaline.	—	--	

TABLE IX.

Dog 8. Weight 14 kilos.
Bled every day at 9 a.m.

Date.	Van Slyke method.		Urine.			Remarks.
	Direct.	Funnel.	Reaction.	Albumin.	Casts.	
1916	*per cent*	*per cent*				
Aug. 5	56	58	Acid.	—	—	
" 6	55	58	"	—	—	After bleeding, 280 cc. of 10 per cent sodium bicarbonate were given.
" 7	60	63	Alkaline.	—	—	

TABLE X.

Dog 3. Weight 13.5 kilos.

	Van Slyke method.		Urine.			Remarks.
	Direct.	Funnel.	Reaction.	Albumin.	Casts.	
	per cent	*per cent*				
Average.....	56	63	Acid.	—	—	
	69	71	Alkaline.	—	—	2 hrs. after 135 cc. of 10 per cent sodium bicarbonate were given.

TABLE XI.

Dog 4. Weight 19 kilos.

	Van Slyke method.		Urine.			Remarks.
	Direct.	Funnel.	Reaction.	Albumin.	Casts.	
	per cent	*per cent*				
Average.....	58	64	Acid.	—	—	
	66	70	Alkaline.	—	—	2 hrs. after 190 cc. of 10 per cent sodium bicarbonate were given.

DISCUSSION.

These investigations show that the nephritis produced by means of uranium nitrate presents a diminution of the plasma carbon dioxide content, associated with an increase of blood urea and plasma chlorides and the appearance of albumin and casts in the urine. These changes indicate the presence of an acidosis in the nephritis produced by uranium nitrate.

Moreover, both the nephritis thus produced and the acidosis which accompanies it can be diminished by means of sodium bicarbonate. In dogs receiving sodium bicarbonate and given uranium nephritis, there is maintained a higher plasma carbon dioxide content, a less pronounced increase of chlorides in the blood, as well as

a diminution of albumin and casts in the urine as compared with animals given uranium nephritis and receiving no soda. In severe nephritis the amount of urea is also diminished in the carbonate dogs as compared with the controls. The nephritis of the carbonate dogs is less severe as regards the histological picture than that of the controls.

CONCLUSIONS.

1. The presence of an acidosis in dogs with experimental uranium nephritis is demonstrable by the Van Slyke-Stillman-Cullen method and that of Marriott. It is detected more readily by the former method.
2. This acidosis is associated with increase in the blood urea and plasma chlorides and with the appearance of albumin and casts in the urine.
3. The oral administration of sodium bicarbonate diminishes the acidosis, the increase in plasma chlorides, the amount of albumin and casts in the urine, and, to a lesser degree, the increase in the blood urea following the administration of uranium. It also diminishes the severity of the changes produced by uranium in the kidneys.
4. The oral administration of sodium bicarbonate to normal dogs raises the carbon dioxide content of the plasma as determined by the Van Slyke-Stillman-Cullen method.

I wish to thank Dr. J. Harold Austin for his constant suggestions and interest throughout the course of this investigation, and Dr. Herbert Fox, Director of the William Pepper Clinical Laboratory, for extending to me the privileges of the laboratory.

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EXPLANATION OF PLATES.

PLATE 57.

FIG. 1. Dog 1. Section of the kidney of a dog with uranium nephritis, to which no soda was given.

FIG. 2. Dog 2. Section of the kidney of a dog with uranium nephritis, to which no soda was given.

PLATE 58.

FIG. 3. Dog 4. Section of the kidney of a dog with uranium nephritis, to which soda was given.

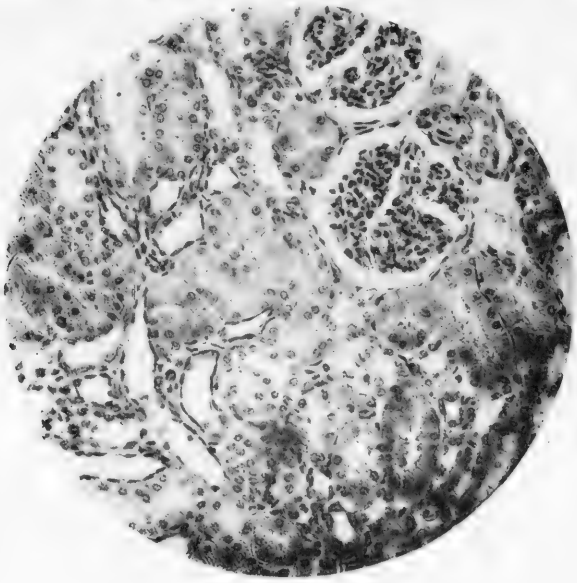

FIG. 1.

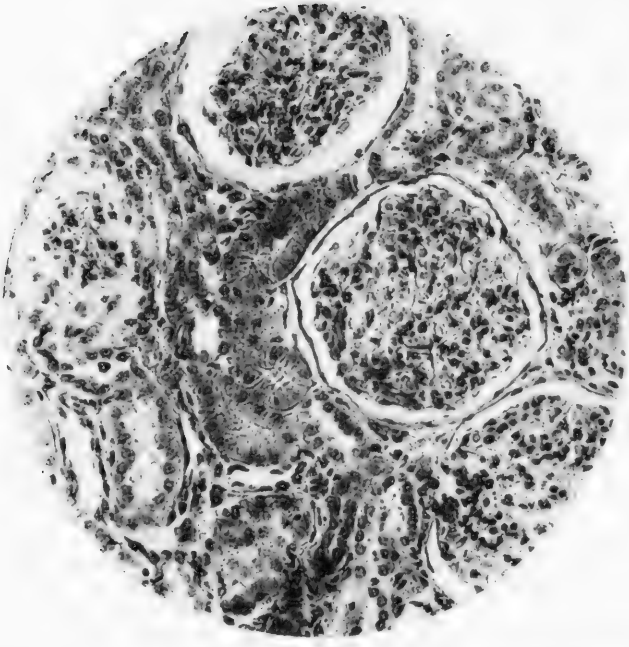

FIG. 2.

(Goto: Uranium Nephritis in the Dog)

FIG. 3.

(Goto: Uranium Nephritis in the Dog.)

CICATRIZATION OF WOUNDS.

IV. Mathematical Study of the Extrapolation Formula and of the Curve of Cicatrization.

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(Received for publication, August 14, 1916.)

Several methods may be used to express the form of the curve of cicatrization by an equation, depending on whether the index i is or is not introduced into the calculation.[1] In such a curve, during the short time, dt, the area cicatrized, ds, remains proportional to the area, S, of the wound, which is expressed:

$$ds = Ks\,dt$$

If S_o is the initial area, K being a constant, integration gives:

$$T = \frac{1}{K} L \frac{S_o}{S}$$

We can now compute the values of $\frac{1}{K}$, corresponding to the observed numbers of S and T. These values increase regularly, and remain finite. Another factor must, therefore, intervene in the process of cicatrization. If this factor is the perimeter, and if we assume that K increases, the relation between K and the perimeter P is:

$$K = \frac{1}{K_1 + K'P}$$

If we assume that the wound does not alter its form as it cicatrizes,

$$\frac{P}{\sqrt{S}} = K''$$

[1] Carrel, A., and Hartmann, A., *J. Exp. Med.*, 1916, xxiv, 429. du Noüy, P. L., *ibid.*, 1916, xxiv, 451, 461.

from which, if $K_2 = K' K''$

$$K = \frac{1}{K_1 + K_2\sqrt{S}}$$

which gives the differential formula:

$$ds = \frac{1}{K_1 + K_2\sqrt{s}} Sdt$$

and by integration:

$$T = K_1 \text{Log}_e \frac{S_o}{S} + 2K_2(\sqrt{S_o} - \sqrt{S})$$

This should represent the curve.[2]

Tables I and II show the results of a few calculations. In the equation the two coefficients K_1 and K_2 remain nearly constant for the same wound. This formula may therefore be considered as representative.

TABLE I.

Patient 263.

Time (T)	Area (S)	Log S	Log $\frac{S_o}{S}$	$\sqrt{S}$	$\sqrt{S_o}-\sqrt{S}$	$T=26 \log \frac{S_o}{S}+2.6(\sqrt{S_o}-\sqrt{S})$
days	*sq. cm.*					
0	61.8	1.791		7.86		$K_1=26$, $K_2=1.3$
4	51.0	1.707	0.084	7.13	0.73	4.06
8	41.6	1.619	0.172	6.45	1.41	8.1
12	33.6	1.526	0.265	5.80	2.06	12.2
16	26.9	1.430	0.361	5.19	2.67	16.3
20	21.3	1.328	0.463	4.62	3.24	20.2
24	16.76	1.224	0.567	4.10	3.76	24.4
28	13.09	1.117	0.674	3.62	4.24	28.6
32	10.12	1.0051	0.786	3.18	4.68	32.5
36	7.77	0.890	0.901	2.79	5.07	36.6
40	5.94	0.774	1.017	2.44	5.42	40.5
44	4.5	0.653	1.138	2.12	5.74	44.2
48	3.4	0.531	1.260	1.84	6.02	48.3
52	2.53	0.403	1.388	1.59	6.27	52.0
56	1.88	0.274	1.517	1.37	6.49	56.4
60	1.38	0.140	1.651	1.175	6.685	60.3
64	1.01	0.004	1.787	1.005	6.855	64.2
68	0.74	$\bar{1}$.869	1.922	0.860	7.00	68.1
72	0.54	$\bar{1}$.732	2.059	0.735	7.13	72.0
76	0.4	$\bar{1}$.602	2.189	0.633	7.23	75.6

[2] This calculation was made by Mr. de Rufz de Lavison.

TABLE II.

Patient 360.

Time (T)	Area (S)	Log S	Log $\frac{S_0}{S}$	$\sqrt{S}$	$\sqrt{S_0}-\sqrt{S}$	$T=25 \log \frac{S_0}{S}+2.5$ $(\sqrt{S_0}-\sqrt{S})$
days	*sq. cm.*					
0	113.1	2.053		10.6		
4	96.8	1.986	0.067	9.8	0.8	3.6
8	81.6	1.911	0.142	9.02	1.6	7.5
12	67.9	1.831	0.222	8.23	2.4	11.0
16	55.9	1.747	0.306	7.47	3.2	15.6
20	45.5	1.658	0.395	6.75	3.9	19.8
24	36.6	1.563	0.490	6.04	4.6	24.0
28	29.2	1.465	0.588	5.4	5.2	27.7
32	23.1	1.364	0.690	4.8	5.8	31.7
36	18.1	1.258	0.796	4.25	6.4	35.0
40	14.0	1.146	0.907	3.74	6.9	39.9
44	10.8	1.033	1.020	3.28	7.3	43.7
48	8.26	0.917	1.137	2.87	7.8	47.9
52	6.27	0.797	1.256	2.51	8.1	51.6
56	4.75	0.677	1.377	2.78	8.4	55.4
60	3.55	0.550	1.503	1.88	8.7	59.4
64	2.64	0.422	1.632	1.62	9.0	63.3
68	1.95	0.290	1.763	1.397	9.2	66.7
72	1.43	0.155	1.898	1.195	9.4	70.1
76	1.04	0.017	2.036	1.019	9.6	75.0
80	0.75	$\bar{1}$.875	2.178	0.971	9.7	78.7

Other examples, without the details of calculation, are as follows:

Patient 408.

t observed.....	4.0	8.0	12.0	16.0	24.0	40.0	According to the above formula, $K_1=10$, $2K_2=6$
T calculated.....	3.94	8.1	12.3	16.6	24.8	39.3	

Patient 361.

t observed.....	4.0	8.0	12.0	16.0	24.0
T calculated.....	3.4	8.2	12.0	16.1	24.1

Patient 366.

t observed.....	4.0	8.0	12.0	16.0
T calculated.....	4.1	8.1	12.0	16.01

Corrected Formula for Narrow Wounds.

The relation between the length and the width of the wound (the form) seems to play a more important part than the perimeter itself (the length of the outline). To express this in practical form, we have been searching for a formula adapted to long and narrow wounds. In wounds resulting, for instance, from longitudinal incision through muscles, an acceleration of the rate of cicatrization is observed within a few days before complete healing, the gain in time being sometimes 12 or 16 days. It is therefore necessary to introduce a new factor, the action of which was predicted by the calculation. This correction is not necessary in average wounds, because as stated above, the change of rate appears only when the length of the wound is very much greater than its width; in other words, when $\frac{L}{l}$ (L being the length, and l the width) is such that:

$$10 < \frac{L}{l} < 25$$

In this case, as the perimeter is considerably increased in relation to the area, its action becomes then—but only then—important, and this explains why, in the case of ordinary circular or oval wounds, with a more or less hollowed outline, it is unnecessary to pay attention to the individual action of the perimeter.

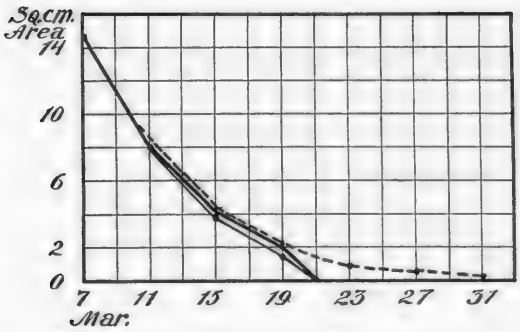
In very long wounds L is practically equal to $\frac{P}{2}$ (P being the perimeter), and may be expressed by $K'\sqrt{S}$. The relation of the outline to the surface then becomes:

$$K'\frac{\sqrt{S}}{S}$$

Experiments showed that when $K' = 1$, this relation is practically equal to $\frac{L}{l}$, and the extrapolation formula becomes:

$$S'' = S'[1 - i(i' + \sqrt{T + i'})] - \frac{\sqrt{S'}}{S'}$$

The following examples show that this equation gives a far better approximation of the date of cicatrization of long and narrow wounds.



TEXT-FIG. 1. Patient 409. Wound of the leg.

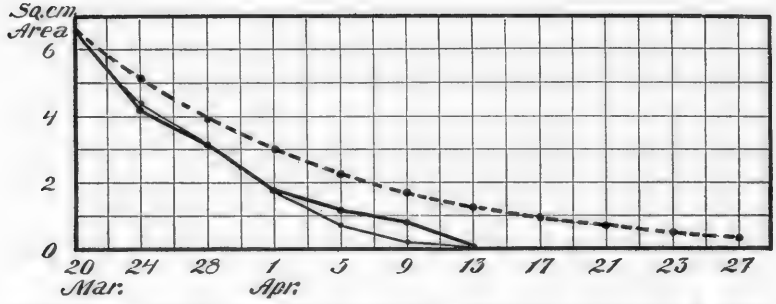
——— observed curve.
——— calculated curve, corrected.
····· calculated curve, uncorrected.

Corrected according to $S'' = S'[1 - i(t' + \sqrt{T + t'})] - \frac{\sqrt{S'}}{S'}$

Uncorrected according to $S'' = S'[1 - i(t' + \sqrt{T + t'})]$

Patient 409 (Text-Fig. 1).

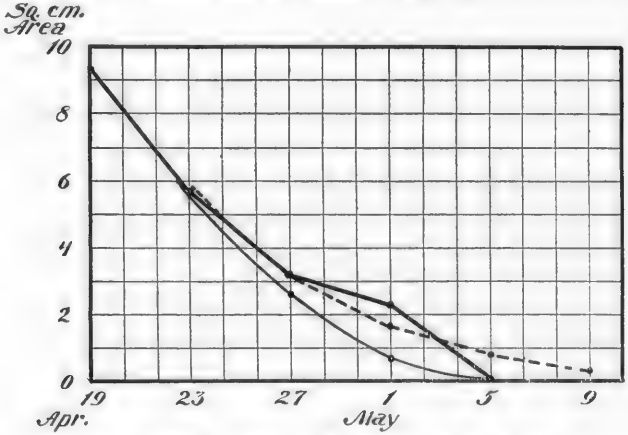
1916 Mar.	7	11	15	19	21	23	27	31
Observed area.....	14.6	8.2	4.3	2.0	0			
Calculated area (corrected).....		8.2	3.95	1.4	0			
" " (uncorrected)....		8.5	4.4	2.1		0.9	0.6	0.4
Length of wound (L) cm.....	13.2	12.3	10.6	7.4				
Width of wound (l) cm. maximum.....	1.6	1.0	0.6	0.3				
$\frac{L}{l}$.....	8.2	12.3	17.6	24.6				



TEXT-FIG. 2. Patient 318. Wound of the leg. Normal rate until Mar. 20, 1916.

Patient 318 (Text-Fig. 2).

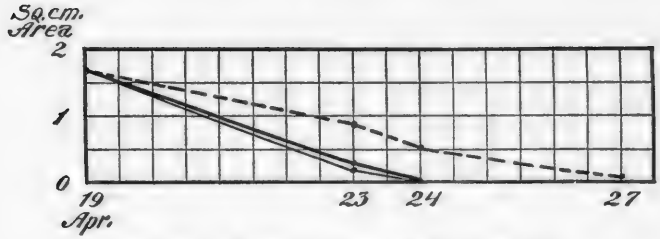
1916 Mar.	20	24	28	Apr.1	5	9	13	17	21	25	27
Observed area	6.5	4.2	3.1	1.8	1.15	0.8	0				
Calculated area (corrected)		4.3	3.1	1.8	0.7	0.2	0				
" " (uncorrected)	6.6	5.1	3.9	3.0	2.25	1.7	1.28	0.95	0.70	0.5	0.37
$\frac{L}{l}$	10.0	10.6	11.6	13.8	17.0	25.0					



TEXT-FIG. 3. Patient 415. Wound of the thigh.

Patient 415 (Text-Fig. 3).

1916 Apr.	19	23	27	May 1	5	9
Observed area	9.3	5.6	3.2	2.3	0	
Calculated area (corrected)		5.5	2.6	0.72	0	
" " (uncorrected)		5.7	3.2	1.65	0.78	0.35
$\frac{L}{l}$	7.5	9.1	12.8	13.4		



TEXT-FIG. 4. Patient 415. Wound of the leg.

Patient 415 (Text-Fig. 4).

1916 Apr.	19	23	24	27
Observed area	1.7	0.3	0	
Calculated area (corrected)		0.22	0	
" " (uncorrected)		0.9	0.5	0.2
$\frac{L}{l}$	9.0	12.5		

As the acceleration of the rate of cicatrization often occurs suddenly, the curves drawn by means of the above formula merely give a more exact determination of the date of complete healing. This formula goes to zero, which corresponds to the cicatrization.

As the perimeter becomes important only when the relation of $\frac{L}{l}$ is above 10, the wound generally cicatrizes according to the uncorrected formula, until it suddenly begins to decrease in size. As soon as the relation between the two extreme dimensions (length and width) reaches a number between 10 and 20, the perimeter apparently becomes important, and reduces the time which should have been necessary for the cicatrization under ordinary circumstances. The fact that both epithelial borders are close to each other probably plays an effective part in this phenomenon.

The time taken is always 4 days.[3]

The corrected and uncorrected formulas can be simplified by expressing the calculated area of a wound after a certain period, as $n \times t$ days. If $t = 4$, $nt = 4n$.

[3] du Noüy, *J. Exp. Med.*, 1916, xxiv, 451.

If the first calculation is 1, the second 2, the third 3, and so on, up to the n^{-1} and the unknown nth; then the area S_n, at the end of $n \times t$ days will be

$$S_n = S_{n-1}\,[1 - i\,(t + \sqrt{nt})],$$

and the correction

$$-\frac{\sqrt{S_{n-1}}}{S_{n-1}}$$

or, if $t = 4$,

$$S_n = S_{n-1}\,[1 - i\,(4 + \sqrt{4n})]$$

THE DIGITALIZED DOG'S HEART AS AFFECTED BY AMYL NITRITE OR ATROPINE, STUDIED ELECTROCARDIOGRAPHICALLY.

By J. T. HALSEY M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

Plates 59 to 65.

(Received for publication, December 18, 1916.)

INTRODUCTION.

This investigation was undertaken with the object of determining the action of amyl nitrite and of atropine on auriculoventricular heart block as produced by digitalis or allied substances. In order to do this it was essential first to work out a method of producing this block with a fair degree of regularity, and also to study the effects of amyl nitrite and of atropine on the normal electrocardiogram.

The accomplishment to a satisfactory degree of the first of these prerequisites was prevented by a number of factors. Chief of these was the tendency in normal dogs to sinus arrhythmia, resulting from the constantly changing vagus and sympathetic tone with its consequent sudden and frequent changes in the rate, rhythm, and conduction time, and the fact that in dogs central vagus stimulation often results in sinus inhibition or block, rather than in auriculoventricular block. As all the drugs used in this investigation exert direct or indirect stimulating or depressing actions on both the vagal and sympathetic control of the heart's function, this constantly occurring normal change in the activity of these two mechanisms proved disturbing and must always be considered in the interpretation of the various phenomena observed.

Methods.

The observations are based on thirty-eight experiments in thirty-four animals. In almost all the experiments dogs were used. Three attempts were made to produce auriculoventricular block in cats,

but these were entirely unsuccessful, strophanthin, although slowly and cautiously given, causing none of the usual vagus effects. This failure probably occurred because the cats were restless throughout the experiment, so that the accelerator mechanism from the start maintained complete control of the circulation (Straub (1)).[1]

In the earlier experiments, after taking control curves, light ether anesthesia was induced, a cannula was inserted into the femoral vein, and after waiting until the electrocardiogram appeared to have returned to its original form, the various solutions were injected and curves taken at short intervals. In later experiments the exposure of the vein was made under cocaine-adrenalin anesthesia and no ether was used. This method appeared to possess certain advantages, among which may be mentioned the fact that it was thus possible to avoid the alteration of the normal rate and rhythm of the heart, which ether always causes, and which frequently persisted for an hour or more after cessation of its administration. Control observations showed that in the dosage employed (0.5 to 1 cc. of cocaine, 1 : 400, and of adrenalin chloride, 1 : 30,000) these drugs produced no detectable alteration in the electrocardiogram. The blood pressure in a number of experiments was recorded by means of a Harvard membrane manometer inserted into the femoral artery.

In a few experiments, in order to keep the animals still, so that sudden and irregular alterations in the circulation might be avoided, a light but persistent narcosis by the administration of luminal sodium was induced.

In doses of 0.04 to 0.06 gm. per kilo by mouth or subcutaneously this drug usually caused a quiet drowsy or stuporous condition, persisting for 24 hours or longer. At times, however, it caused a marked lowering of the blood pressure and persistent rapid pulse and appeared, in some experiments, to prevent the development of the usual digitalis actions. Its use was, therefore, abandoned. The usual effects of its administration were an increase in the heart rate and an abolition of the sinus arrhythmia which is almost invariably present in the quiet dog. After 36 to 48 hours these effects passed off and the heart returned to its original action. No effects on the electrocardiogram were noted other than were due to the above, which would naturally result from a diminution of the vagus tone, such as is to be expected from large doses of any of the lipoid-soluble narcotics.

[1] Straub (1) reports similar results with these animals.

It is important to report that in four of the thirty-four dogs used in these experiments a spontaneous auriculoventricular block was observed. At The Rockefeller Institute for Medical Research in the past 6 years the same phenomenon has been observed in at least four additional cases. The occurrence of this condition, by no means rare, should be remembered by those using these animals, and should lead to caution in interpreting the significance of its appearance. An example of this is seen in Fig. 1, A (Experiment 85).

All observations were made with Lead II. As a majority of the animals survived, control curves were in many instances taken on the following day.

The Effects of Strophanthin and Digitalis on the Normal Dog's Heart.

Strophanthin.

Dosage.—Crystalline strophanthin was injected into the femoral vein in amounts ranging from 20 to 95 per cent of the average lethal dose (1.01 mg. per kilo, according to Jamieson (2)), using a solution of which 1 cc. contained 0.02 mg. of Thoms crystalline *g*-strophanthin. In the majority of the experiments from 30 to 40 per cent of the lethal dose was injected in about 15 minutes. It was found that the dose which produced a marked vagus effect, as shown by pronounced slowing, auricoul entricular block, or exaggerated sinus arrhythmia, usually lay between 30 and 40 per cent of the lethal dose. Doses above 40 per cent of the lethal dose were likely to produce ectopic ventricular beats or acceleration of the sinus rate, or both. As in many of the experiments the observations extended over periods of several hours and as the various strophanthin effects pass off more reness rapidly, these effects were maintained in a number of experiments by intermittent slow injection of very dilute solutions. The majority of the animals recovered promptly, often within a few hours, from doses up to 60 per cent of the minimum lethal dose.

Rate (*Excluding Changes Due to Ectopic Contractions*).—In twelve of the nineteen experiments the administration of strophanthin was followed by slowing (Table I, Group A), in four by slowing followed by acceleration (Table I, Group B), and in three by acceleration without previous or subsequent slowing (Table I, Group C).

In interpreting the significance of acceleration of the sinus rate, it is impossible to exclude the influence of psychic factors, but in this series as well as in the digitalis series, reported below, there were a number of instances in which, as far as could be determined by close observation of the dog, the acceleration did not appear to be preceded or accompanied by any evidence of excitement or unrest. The acceleration noted is consequently attributed to a direct action on the sinus node, such as was observed by Rothberger and Winterberg (3) in their experiments on dogs in which the cardiac nerves had been cut.[2]

Rhythm.—The initial rhythm was affected as follows: auriculoventricular block resulted in nine of nineteen experiments. In one of these (No. 52) it was present in the control curve, was abolished by amyl nitrite, and returned for a short time after strophanthin had been injected. In only two experiments, Nos. 56 (Fig. 2, B) and 57, were fairly persistent auriculoventricular blocks obtained, but in six others occasional blocks were produced.[3] In nearly all these cases the sinus arrhythmia was markedly exaggerated by strophanthin. Occasional sinus block was produced in two experiments with interauricular (P-P) intervals ranging up to 1.57 seconds. The sinus arrhythmia was exaggerated by strophanthin in fourteen experiments, but was little or not at all affected in three, and was lessened in two experiments.

Auriculoventricular (P-R) Interval.—This was variously affected by the administration of strophanthin. At some time in the majority of the experiments (fourteen out of nineteen), it was more or less lengthened, on the average by 17 per cent of the initial conduction time. At times this effect was very pronounced, as for example in Experiments 56, 59, and 63, where it was increased from averages of 0.118, 0.112, and 0.116 before, to averages of 0.152, 0.145, and 0.146

[2] Under the conditions in my series of experiments a direct stimulation of the central accelerator mechanism may also occur and is not impossible in view of the known stimulation of another closely related sympathetic (the vasomotor) center.

[3] In the endeavor to secure the desired auriculoventricular block, the rate of injection, the size of the dose, and the concentration of the solutions injected were varied in different instances, but without success.

TABLE I.

Effect of Strophanthin on Sinus Rate in Dogs.

Group.	No. of experiment.	Weight.	Maximum effect on rate.		Lethal dose given.	Duration of administration.	Remarks.
			Before.	After.			
		gm.			*per cent*	*min.*	
A	52	5,000	180	140	40	14	A–V block.
	54	7,500	110	76	60	116	Pronounced S–A.
	56	6,250	140	84	40	13	A–V block.
	57	6,900	109	65	35.7	17	Sinus block preceded by A–V block.
	58	6,250	120	54	54	164	Sinus block.
	60	6,000	80	65	35	12½	Ectopic beats before and after strophanthin.
	62	8,800	80	72	38	180	
	63	6,600	120	96	30	13	
	64	7,750	92	60	40	55	
	65	8,250	162	96	37	56	Dog had had atropine 87 min. before rate of 162.
	67	8,600	140	80	37	65	Dog had had atropine 60 min. before rate of 140.
	69	11,800	70	60	35	29	Second strophanthin injection started 205 min. after first strophanthin injection and 140 min. after atropine.
			120	60	Additional 20	20	
B	59	7,000	110	100	25	5	This dog was restless.
				235	40	10	A few ectopic beats occurred (7 ectopic to 50 nomotopic) when this rate was reached.
	61	8,500	120	90	24	7	The acceleration in this experiment occurred so late (24 min. after the dose of 35 per cent) that it may not have been due to strophanthin.
				190	35	15	
	71	10,000	145	130	25	9	
				155	30	13	
	73	6,700	130	105	34	14	
				145	55	42	
C	55	8,000	102	235	50	16	Following the strophanthin ectopic beats persisted most of the time for 100 min., but the rate of 235 is without ectopic beats.
	68	7,200	150	240	30	12	Atropine 135 min. earlier.
	72	9,750	140	240	56	30	

The apparent discrepancy as to frequency of A–V blocks between the table and the text (page 732) is due to the fact that A–V blocks are mentioned under "remarks" only when present at, or near the time when minimum figures occurred.

seconds after the administration of strophanthin. In the three experiments the longest intervals before giving strophanthin were 0.14, 0.14, and 0.13 seconds; after it they were 0.19, 0.165, and 0.21 seconds. In Experiment 62 the longest P-R interval in the control curves was 0.18 second, but after strophanthin there was one of 0.37 second. In two experiments the P-R interval was first lengthened, then shortened; in two others first shortened then lengthened; and in one it was never lengthened, but was slightly shortened from an average of 0.10 to 0.094 second, the sinus rate varying only from 92 to 95 per minute.

In many instances the change in conduction time was obviously influenced by the change in sinus rate, and in many others the changes noted were slight. But there is no doubt that as a general rule the P-R time was lengthened by strophanthin. Pronounced shortening of the conduction time was, however, observed at times in experiments in which it could not be attributed to change in the sinus rate, but would appear to have been due to sympathetic stimulation, either central, from psychic cause, or as a result of direct drug action, or to drug action on the conduction paths themselves.[4] An example of this is Experiment 64 in which the P-R time originally varied from 0.09 to 0.11 second, but after a dose of strophanthin it ranged from 0.10 to 0.17 second, and then after a second dose ranged between 0.07 and 0.10 second, the sinus rate varying only from 80 to 78 to 82 per minute.[5]

P Wave.—In the majority of the experiments (fourteen out of nineteen) the P wave showed no significant changes. In the five others the administration of strophanthin was followed at certain stages by a lessening of the height of this wave, which varied from strongly positive to isoelectric and occasionally became diphasic. In one of these experiments occasional negative P waves were noted. As the height of the P waves varies spontaneously in dogs from time to time and may even be negative in control curves, only a distinct increase or decrease has been considered to be significant. A lessen-

[4] For the latter mode of action these experiments produce no evidence, nor is it known to me that an analogous action is exerted by any other drug.

[5] The quiet behavior of this dog and the lack of increase in the sinus rate speak strongly against psychic stimulation as a cause of this effect on the P–R time.

ing of its height (Einthoven, and Einthoven and de Waart (4))[6] or a change to negative appears to result under certain conditions from increased vagus tone, but there are of course other possible explanations for this change in the wave (Rothberger and Winterberg (5)).[7]

Ventricular Complex (*R and T Waves*).—This frequently showed changes (in sixteen out of nineteen experiments). Ectopic ventricular contractions, usually of the left ventricular type, appeared after as little as 19, 25, 30, 31, and 33 per cent of the lethal dose, and almost invariably occurred if more than 40 per cent of the lethal dose was given in about 15 minutes. After the larger doses these ectopic beats were of many types. While nomotopic cycles persisted, the T wave was commonly altered by even smaller doses. Slowing of the sinus rate appeared to exert some influence in favoring the occurrence of ectopic beats, while acceleration appeared to hinder it. In certain experiments large amounts (*e.g.*, Experiment 73, where 70 per cent of the minimum lethal dose was given in 67 minutes) failed to cause ectopic contractions.

As the effective vagus stimulating dose in dogs appears to lie between 20 and 40 per cent of the lethal dose, it is a matter of interest and perhaps of some importance that ectopic ventricular beats are so frequently produced by these doses. In Experiment 56 after 40 per cent of the lethal dose had been given in 13 minutes auriculoventricular block appeared (Fig. 2, B). The ventricular contractions were ectopic in origin, possibly as the result of the stimulation of tertiary centers by strophanthin, which had been previously injected. In all the experiments in which ectopic contractions followed the administration of strophanthin, they appeared during or shortly following its injection, with the exception of certain experiments which will be discussed below, and in which their more tardy appearance can be attributed to the action of amyl nitrite or atropine. In Figs. 3, D, 4 B, D, and E, 5, B, C, D, and E, and 6, G the various types of ectopic complexes noted may be seen.

[6] This was observed by Einthoven and de Waart (4) after stimulation of the vagus.

[7] For example, Rothberger and Winterberg (5) obtained negative P waves by simultaneous stimulation of the vagus and accelerator nerves and at times by stimulation of the accelerator alone.

The R wave, as long as nomotopic cycles were present, as a rule underwent no significant alterations in shape. In a few experiments it became much shorter.

The T wave changes were difficult to analyze, as this wave is variable in the normal controls and as it changes rapidly and markedly with changes in rate and rhythm. The change noted by Cohn, Fraser, and Jamieson (6) as characteristic for digitalis in human beings, *i.e.*, a change from one sign to the other, appeared in the majority of the cases where vagus effects (slowing, increase in sinus arrhythmia, etc.) occurred, and was also often present before these effects were apparent. On the other hand, marked vagus effects were at times apparent without the characteristic effect on the T wave. Frequently, especially where there was acceleration of the heart rate with or without the occasional occurrence of ectopic contractions the T wave became decidedly taller (Fig. 7, B), an alteration observed by a number of authors (Selenin (7), Nicolai and Simons (8), Straub (1), and Rothberger and Winterberg, and Einthoven and de Waart (9)), and considered, apparently correctly, by the four last named as the result of a direct stimulation of the sympathetic nerve structures (tertiary centers) in the ventricles.[8]

Digitalis.

The infrequency with which strophanthin produced persistent auriculoventricular block suggested the use of the entire digitalis body instead of the single active principle. For this reason digipuratum was administered in eleven experiments and a fluid extract of digitalis (Parke, Davis and Company) was used in three others. It is customary in therapeutics, no doubt with justice, to regard strophanthin as more easily absorbed by the heart muscle and its action as more prompt, but at the same time more transitory, than that of digitalis. It was therefore presumed that when digitalis was administered intravenously to dogs, the alteration of rhythm would come on more slowly and more uniformly and would be more

[8] In my experiments a direct stimulating effect on the accelerator (or sympathetic) centers may be involved, but Rothberger and Winterberg (9) observed identical changes following the administration of strophanthin in dogs after bilateral vagotomy and destruction of the stellate ganglia.

persistent than had been the case with strophanthin. The use of these bodies was, however, attended by no greater success than when strophanthin was used. Contrary to expectation, the effects produced by digitalis[9] ensued as promptly and passed off as rapidly as had been the case with strophanthin. The digitalized heart also reacted to amyl nitrite and to atropine just as did the strophanthinized heart. Apparently in the dog digitalis is rapidly excreted or in some other way rendered inactive. These observations are in accord with the fact that these animals are highly tolerant of digitalis by mouth and with the experience that it has been difficult to produce in them the ordinary digitalis effects by oral administration. Briefly summarized, the effects usually produced by digitalis or strophanthin under the conditions obtaining in this series were as follows: slowing of the sinus rate, increased conduction time, occasional auriculoventricular blocks, or exaggerated sinus arrhythmia. The T wave in many experiments became negative, but often was unaltered or became more positive.

Ectopic ventricular beats of the left ventricular type frequently followed the administration of doses not exceeding 40 per cent of the minimum lethal dose. Doses larger than this usually caused the occurrence of ectopic ventricular contractions, which at first were of the left ventricular type but later were of various sorts.

The Effects of Amyl Nitrite and of Atropine on the Normal and Digitalized Dog's Heart.

Amyl Nitrite.

Amyl nitrite has been employed clinically (Josué and Godlewski (10), Petzetakis (11), and Belloir and Dubos (12)) as a means of determining whether an auriculoventricular block is due to central vagus stimulation or to direct action on, or organic changes in the conduction paths. Its power of abolishing heart block or of accelerating the sinus rate, according to our present knowledge of its pharmacology, results from its ability to cause a fall of the blood pressure, which in turn depresses the vagus and stimulates the accelerator center.

[9] Twelve frog units per kilo were taken as the minimum lethal dose.

Earlier investigators of this drug concluded that it had a marked early stimulation effect on two of the important medullary centers, the vagus[10] and the respiratory, the action on the vagus being attributed by them to reflexes from the upper air passages (Filehne (13) and Brunton (14)). As it has been shown by Pilcher and Sollmann (15) that a third medullary center, the vasomotor, is stimulated even when these reflexes are excluded and as its similar action on the respiratory center is universally recognized, it is possible that the vagus center is also acted on in the same way; *i.e.*, direct stimulation.

The depression of vagus tone which is subsequently seen is due to the fall in blood pressure which amyl nitrite causes and which removes the physiological stimulus for vagus activity. This conception of the cause of the changes in vagus tone during the inhalation of amyl nitrite is in accord with the phenomena observed in this series and accounts for the sequence of events about to be described.

Rate.—In normal, strophanthinized, and digitalized dogs the rate was often slowed at first by amyl nitrite, at times strikingly; as for example in Experiment 53 (Fig. 8, B), in which, after inhalation for 70 seconds, the rate fell from 66 to 42, and Experiment 64 where after inhalation for 20 seconds the rate fell from 72 to 56. This slowing occurred only in dogs in which the vagus was already exerting a marked effect, as evidenced by the slow rate, or auriculoventricular block, or sinus arrhythmia. As a rule, with continued inhalation the rate was markedly increased. In Experiment 53, for instance, the rate rose later to 195 per minute (Fig. 8, C).

The primary slowing is undoubtedly due to central vagus stimulation, direct or indirect. It was followed by acceleration, provided

[10] Filehne (13), for instance, reports an experiment in which the inhalation of amyl nitrite by a rabbit caused a fall in the heart rate from fifty-three to ten beats per 15 seconds. Pharmacologists have more recently apparently overlooked this early vagus stimulating effect, probably because in ordinary laboratory demonstrations the drug is inhaled through a tracheal cannula. This method, of course, minimizes reflex respiratory effects, while the anesthesia universally employed renders the vagus center less sensitive to stimulation either direct or indirect. I have occasionally seen a primary slowing of the heart rate of slight degree and short duration from amyl nitrite, even in tracheotomized and anesthetized animals. As this investigation was undertaken in the hope of obtaining information bearing on the clinical use of this drug in studying heart block and other arrhythmias, no experiments were done in which the drug was administered by tracheal cannula. Consequently in the majority of the observations made the effects of this vagus stimulation were very apparent.

enough amyl nitrite was given to cause vasodilation sufficient to affect the blood pressure, as was seen in all the observations in which the blood pressure was recorded. The curves show the reciprocal relation between the rise and fall in the blood pressure and the increase or diminution of vagus tone as indicated by the heart rate. These relations may be seen in Fig. 1, B to G. In several experiments where the blood pressure was not recorded even prolonged inhalation of amyl nitrite failed to cause acceleration of the heart rate, but the numerous records where the blood pressure was recorded show no instance where, in spite of a fall in the blood pressure, the heart rate was not accelerated markedly.

Rhythm.—As a rule, the rhythm was promptly and decidedly affected by the inhalation of amyl nitrite, sinus arrhythmia or auriculoventricular blocks, whether spontaneous or due to drugs, being (if the drug was given in sufficient doses) abolished or at least markedly lessened (Figs. 1, A, B, D, E, and G, and 8, B and C). In a number of the experiments, however, the effects were preceded by an exaggeration of the arrhythmia due to the vagus stimulation already referred to, and in a number of instances inhalation lasting for several minutes failed completely to abolish the arrhythmia or blocks. An illustration of this early vagus stimulation is seen in Fig. 8, B.

Especially striking was the vagus stimulation in Experiment 53 (Fig. 8, B) in which the interventricular (R–R) interval, which had previously ranged from 0.54 to 1.34 seconds, varied from 0.78 to 4.02 seconds during an inhalation lasting 70 seconds. In this experiment the sinus arrhythmia was not abolished until amyl nitrite had been inhaled for 7 minutes and 40 seconds. In Experiment 64 sinus block (or pronounced sinus arrhythmia) which occurred after strophanthin was given was not abolished by an inhalation lasting 140 seconds, but was abolished later by one lasting 20 seconds. In Experiment 52 a spontaneous auriculoventricular block was not abolished by the first inhalation lasting 60 seconds but was abolished by a second one lasting 90 seconds. In Experiment 56 amyl nitrite inhalation twice failed to abolish an auriculoventricular block due to strophanthin, although the inhalation was continued the second time for as long as 5 minutes. Unfortunately in all the observations cited above the blood pressure was not recorded.

In a number of other experiments in which the blood pressure was recorded, similar but less extreme vagus stimulation occurred, but later gave way to vagus depression (and probably central accelerator stimulation as well) when the vasodilating effect of amyl nitrite became sufficiently pronounced. Fig. 1, A to G

illustrates these effects, both before and after the administration of digitalis to a dog in which there was a spontaneous auriculoventricular block.

In view of the fact that whenever amyl nitrite produced sufficient vasodilation, as seen in the blood pressure records, it also invariably abolished or lessened the arrhythmia or blocks, it may be concluded that its failure to relieve the irregularity in a number of the earlier experiments was due to insufficient dosage.

P-R Interval.—The P-R interval was markedly altered by amyl nitrite. As a rule, the first effect during the stage of vagus stimulation was to lengthen the interval, but later, when a sufficient amount of the drug had been inhaled, the conduction time was markedly lessened, often to 50 to 60 per cent of its original length, while at the same time acceleration of the heart rate took place. These changes are well shown in Experiment 62 in which the P-R time originally varied from 0.09 to 0.16 second. After the inhalation of amyl nitrite for 15 seconds it ranged from 0.11 to 0.56 second, and after 60 seconds was shortened to a uniform length of 0.08 second.

P Wave.—The P wave was frequently markedly altered by amyl nitrite, usually becoming more positive after its inhalation (Figs. 6, B and C, and 7, B, C, and D). In Experiment 62 in which after the administration of strophanthin this wave varied from an isoelectric to a positive form, all P waves became strongly positive during the inhalation of amyl nitrite (Fig. 9, B, C, and D). This change in the P wave corresponds to the change in the form of this wave under the influence of sympathetic stimulation as described by Rothberger and Winterberg (5).

Ventricular Complex.—The ventricular complex, as long as it represented a normally contracting ventricle, often showed significant changes in the T but not in the R wave after amyl nitrite inhalation. The former was frequently altered by amyl nitrite, generally as follows: During the stage of vagus stimulation it often became less positive, but later, as a rule, when the vagus control had been abolished by this drug the T wave became markedly positive (Fig. 7, B, C, and D). When digitalis or strophanthin in not too large amounts had caused the appearance of ectopic beats, amyl nitrite almost always caused their temporary or permanent disappearance (Fig. 4, C). Even after very large doses of strophanthin or

digitalis had caused a constant succession of ectopic beats, the administration of amyl nitrite usually was followed by the reappearance of occasional normal cycles or by striking alteration in the form of the ventricular complexes so that they more closely resembled that of nomotopic ventricular contractions (Fig. 5, C).

In a single experiment (No. 84), in which 33 per cent of the lethal dose of digipuratum had been given 28 minutes earlier, ectopic ventricular contractions appeared for the first time 2 minutes after an inhalation of amyl nitrite, lasting 90 seconds, had abolished a pronounced sinus arrhythmia, and caused a marked acceleration of the sinus rate. The occurrence of these contractions, originating in the tertiary centers, stimulated as they were by the digipuratum previously given, may have been due to the abolition by amyl nitrite of the vagus tone by removing its negative inotropic action on the ventricle alone, or in combination with stimulation of the accelerator center. This explanation seems improbable in view of the fact that the ectopic beats continued to appear from time to time for 26 minutes after the amyl nitrite had been stopped, and that in many other observations[11] amyl nitrite exhibited a striking power of abolishing ectopic contractions if they were already occurring. On the other hand, in no other experiment did ectopic contractions develop so long after the administration of digitalis or strophanthin. Another factor to be considered in estimating the significance of this isolated observation is the occasional, if rare, spontaneous occurrence in dogs of ectopic ventricular contractions.[12]

In view of the similar reaction of arterial and heart muscle, and of the neuromuscular (intermediate) substance contained therein, to various pharmacological agents (*e.g.*, epinephrin) and physiological stimuli (*e.g.*, stimulation of sympathetic nerve supply), the effects observed in these experiments should have been expected. Because of the close relation of these structures and the known dominant action of amyl nitrite, that is, depression of the sympathetic neuromuscular organs in the arterial walls, it is not surprising that this drug produces a similar depressing action on the cardiac muscle through its neuromuscular (intermediate) substance. It is im-

[11] Later in the same experiment, when 44 per cent of the lethal dose of digipuratum had caused almost constant ectopic contractions, inhalation of amyl nitrite for 120 seconds caused them to disappear temporarily.

[12] In the series of thirty-eight experiments, this was observed but once, and then in a period when the heart rate was slow, which was not the case in this experiment.

portant to emphasize the fact that, in order to produce this depressing action on the tertiary cardiac centers, very large doses were necessary. Otherwise one might be tempted to expect that one could produce analogous effects in human patients by administering amyl nitrite. That this is possible cannot be denied, but one attempt to do so was unsuccessful.[13]

Atropine.

Atropine also has been used by a large number of clinicians in the study of various types of cardiac irregularity, especially in cases of heart block, to determine whether the block is of vagal or other origin, but as far as is known to the writer it has not been studied experimentally under such conditions as obtain in this series. It is for this reason that it appeared desirable to study its effect on the normal and digitalized heart and to compare its action with that of amyl nitrite.

Atropine sulfate was administered intravenously to twelve dogs in doses ranging from 0.004 to 0.07 mg. per kilo. The effects on rate, rhythm, etc., varied markedly with the size of the dose. Small doses often produced the effects of central vagus stimulation; *i.e.*, lengthening of the auriculoventricular interval and auriculoventricular block. The sinus rate, if affected at all, was accelerated. Larger doses usually produced similar results at the start, but were followed quickly by the effects of vagus depression; namely, acceleration of the rate, shortened P-R interval, and abolition of sinus arrhythmia or of auriculoventricular block.

Sinus Rate.—The sinus rate was increased by atropine in each observation in which any change was noted, but in several experiments the smaller doses produced little or no effect. In no case did the repetition or increase in the size of the dose fail to cause marked acceleration of the sinus rate. The general effect of the action on sinus rate is seen in Table II. In the experiments in which the heart was slowed by previous administration of strophanthin or digitalis the rate after atropinization was increased to approximately

[13] This was in a patient with an attack of paroxysmal tachycardia of auricular origin, seen by me through the courtesy of Dr. Lerch in his service at the Charity Hospital in New Orleans.

the same figure as had been reached after similar doses given before strophanthin or digitalis had been administered; that is, the increase in rate was as great after as before digitalization. In the majority of cases, however, the acceleration produced by atropine did not last as long in strophanthinized or digitalized dogs as in the controls.

Rhythm.—The effects on rhythm in twenty-four observations varied with the size of the dose. A small dose usually caused auriculoventricular block, which was abolished by repetition of the dose (Fig. 10, B and C), or if a block was already present, it was first exaggerated and then abolished. For example, in Experiment 62 administration of strophanthin was followed by marked sinus arrhythmia and occasional auriculoventricular blocks. A first injection of 0.016 mg. of atropine sulfate per kilo caused an increase in the frequency of the blocks, and a second dose of the same size abolished both block and sinus arrhythmia (Fig. 9, E, F, G).

Previous administration of strophanthin or digitalis did not appear to produce either qualitative or quantitative changes in the effects produced by atropine.

Auriculoventricular (P-R) Interval.—As was expected, this was markedly affected by atropine. By doses which abolished the arrhythmia or blocks and accelerated the sinus rate it was usually but not always shortened, while after doses causing auriculoventricular blocks it was, as a rule, lengthened. There were, however, many exceptions. Apparently the effect of atropine on the P-R interval was often complicated by the effect which acceleration or retardation of the sinus rate normally exerts in retarding or accelerating the rate of conduction (Table II). As may be seen in a number of instances (Experiments 65, 67, 68, and 69, Table II), and as is illustrated in the chart from Experiment 69 (Text-fig. 1), there is often the same lack of parallelism in the effects on sinus rate and conduction time (P-R interval), to which attention has been called by Cohn and Fraser (16). This may be due either to a quantitatively unequal effect on different parts of the vagus center or on the different vagus nerve endings, the former of which are stimulated while the latter are depressed.

TABLE II.

Effect of Atropine on Conduction Time, Rhythm, and Sinus Rate in Dogs.

Dog No.	Strophanthin previously given.	P-R interval before.	Average.	Atropine per kilo.	P-R interval after.	Average.	Rhythm. Before.	Rhythm. After.	Effect on sinus rate.	Effect on P-R time.
		sec.	*sec.*	*mg.*	*sec.*	*sec.*				
62	+	0.11 -0.16		0.016	0.11 -0.17 0.12 -0.18 0.12 -0.20		S-A and occasional A-V block.	A-V block.	+	Increased.
	+	0.11 -0.16		0.033 in 11 min.	0.10 -0.18 0.11 -0.15 0.11 -0.13		"	Regular.	++	" then diminished.
64	+	0.085-0.10	0.092	0.016	0.08 -0.09	0.084	S-A	S-A	0	Diminished.
	+	0.085-0.10	0.092	0.033 in 2½ min.	0.08 -0.095	0.094	" "	Regular.	+	0
	+	0.085-0.10	0.092	0.05 " 12 "	0.08 -0.085	0.082	" "	"	++	Diminished.
	+	0.11 -0.125	0.115	0.025	0.11 -0.125	0.115	" "	"	++	0
	+	0.105-0.12	0.118	0.025	0.08 -0.095	0.087	" "	"	++	Diminished.
65	0	0.12 -0.14	0.124	0.006	0.12 -0.14	0.13	" "	S-A	+	Increased.
	0	0.12 -0.14	0.124	0.012	0.10 -0.11	0.108	" "	Regular.	++	Diminished.
	+	0.12 -0.13	0.125	0.012 in 3 min.	0.13 -0.15	0.133	" "	"	+	Increased.
67	0	0.11 -0.12	0.114	0.006	0.12 -0.145	0.129	" "	S-A	Slight +	"
	0	0.11 -0.12	0.114	0.012 in 6 min.	0.11 -0.14	0.122	" "	A-V block.	+	"
	0	0.11 -0.12	0.114	0.033 " 21 "	0.09 -0.11	0.10	" "	Regular.	++	Diminished.
	+	0.13 -0.17	0.143	0.006	0.14 -0.16	0.148	" "	Occasional A-V block.	0	Slightly increased.
	+	0.13 -0.17	0.143	0.012 in 10 min.	0.13 -0.17	0.15	" "	Regular.	+	"
	+	0.13 -0.17	0.143	0.02 " 15 "	0.11 -0.115	0.112	" "	"	++	Diminished.

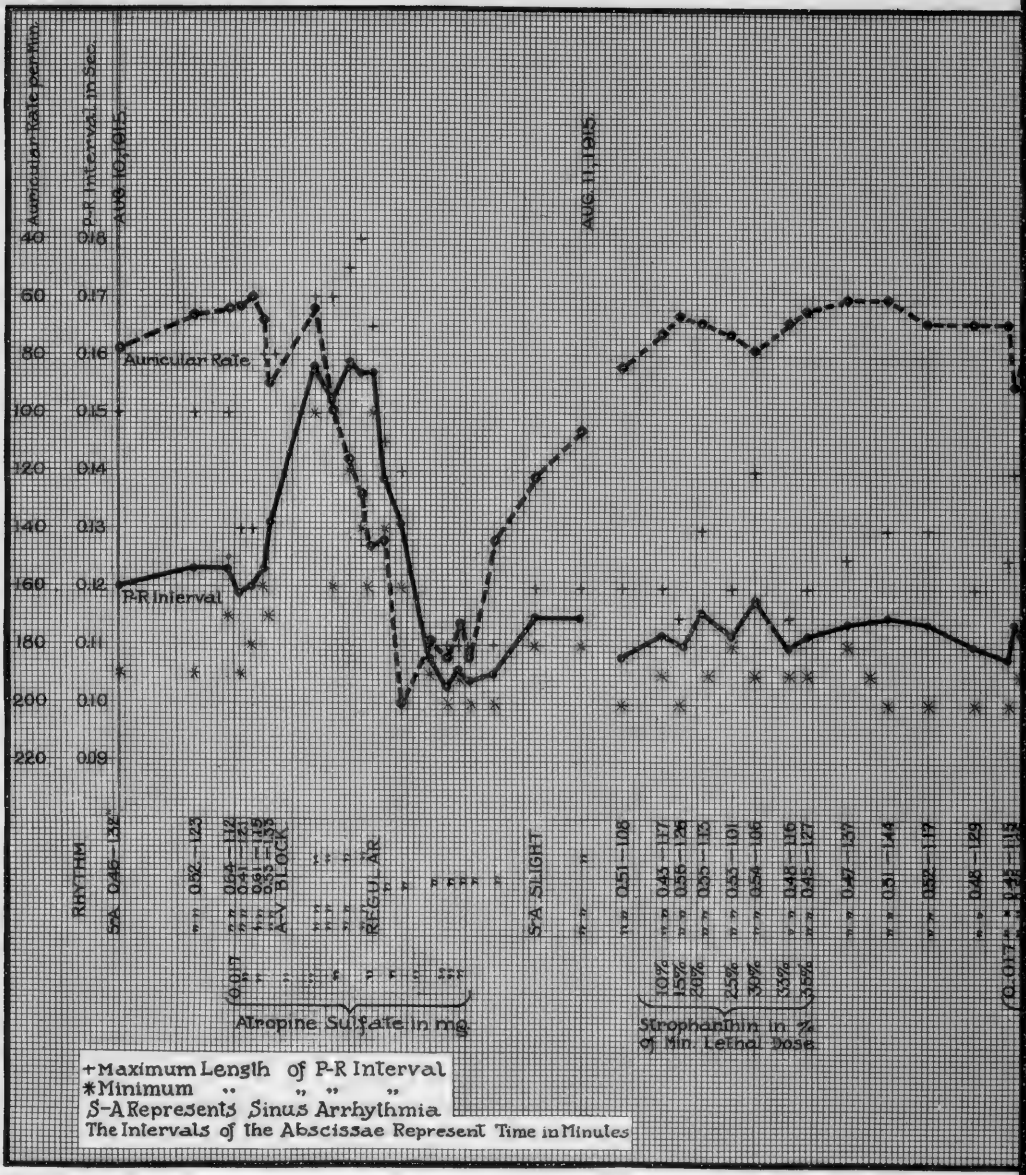
68	0	0.09 –0.10	0.0925	0.007	0.0825–0.16	0.098	S–A	Occasional A–V block.	+	Slightly increased.
				90 sec. later.	0.07 –0.095	0.086	" "	"	+	Slightly decreased.
	0	0.09 –0.10	0.0925	0.013 in 5 min.	0.07 –0.095	0.083	" "	Regular.	++	Diminished.
69	0	0.115–0.15	0.126	0.01 " 2 "	0.11 –0.13	0.119	" "	S–A	0	Slightly diminished.
	0	0.115–0.15	0.126	0.02 " 9 "	0.15 –0.17	0.157	" "	" " and occasional A–V block.	+	Increased.
	0	0.115–0.15	0.126	0.05 " 35 "	0.10 –0.11	0.103	" "	Regular.	++	Diminished.
	+	0.10 –0.125	0.108	0.012 " 5 "	0.105 –0.14	0.115	" "	A–V block.	+	Increased.
	+	0.10 –0.125	0.108	0.016 " 7 "	0.11 –0.20	0.15	" "	S–A, no A–V block.	+	"
	+	0.10 –0.125	0.108	0.05 " 42 "	0.095 –0.10	0.098	" "	Regular.	++	Diminished.

P Wave.—In some of the experiments, but not in all, the P wave underwent distinct modifications under the influence of atropine. Usually when atropine caused an auriculoventricular block the P wave became less positive or isoelectric or negative (Figs. 9, F and 10, B). It became positive again when subsequent peripheral vagal depression manifested itself (Figs. 9, G and 10, C). Doses which caused marked acceleration and regular rhythms usually caused the P wave to become more strongly positive.

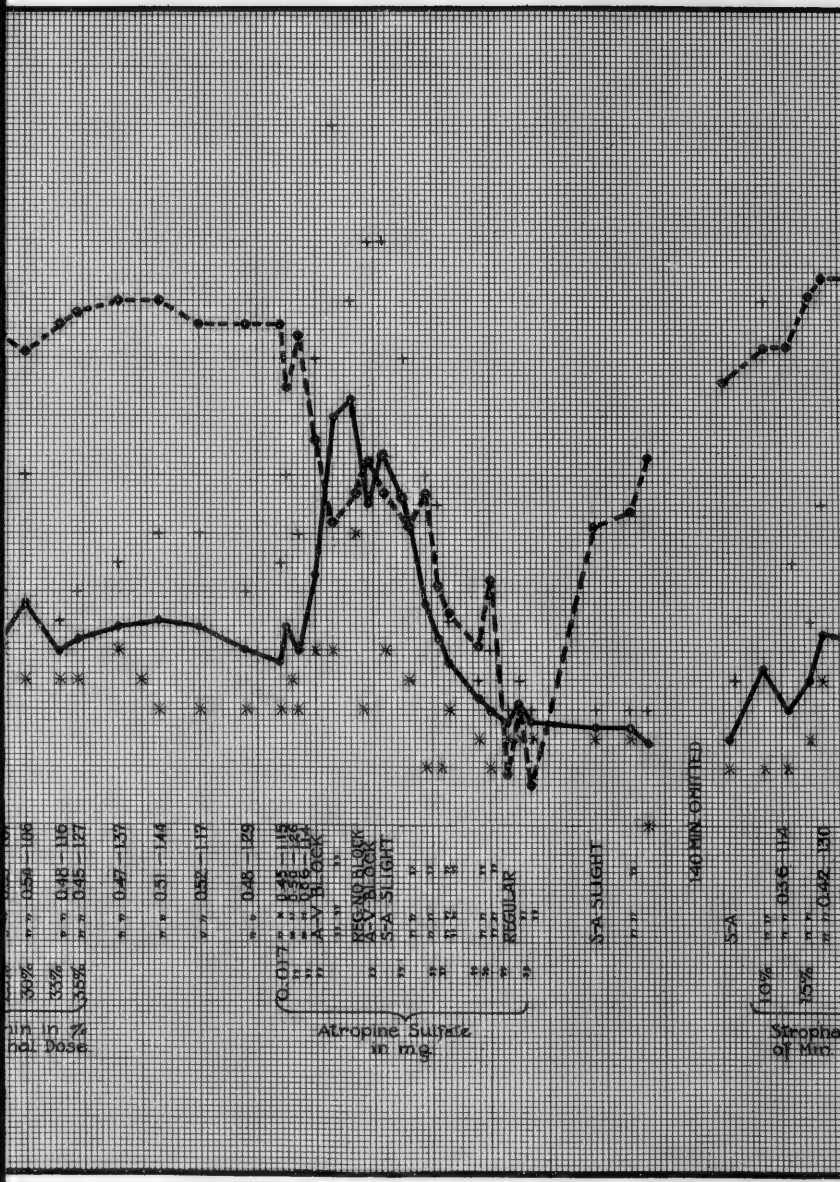
Ventricular Complex.—The ventricular complex was at times altered in various ways. The T wave during the stage of vagus stimulation showed no changes of significance, perhaps because in all such observations the vagus was already acting rather powerfully before atropine was administered. Later, however, when vagus control had been abolished by atropine, the T wave in most instances became decidedly more positive (compare Fig. 6, E with 6, D). When the ventricular complexes were normal before the administration of atropine, no other changes were noted except in two instances.

In Experiment 64, although large amounts of strophanthin (71 per cent of the minimum lethal dose in 4 hours and 29 minutes, and 77 per cent in 5 hours and 14 minutes) had been given, the ventricular complexes were normal. Two injections of atropine, given 48 minutes apart, were each followed by the occurrence of ectopic beats of the right ventricular type, which persisted each time for less than 10 minutes (Fig. 3, D and E). In Experiment 83, 18½ minutes after 39 per cent of the lethal dose of digipuratum had been given and 60 seconds after a second injection of 0.064 mg. of atropine sulfate incomplete heart block appeared (Fig. 6, F). Ectopic ventricular beats appeared 5½ minutes later (Fig. 6, G). A third injection of 0.125 mg. given 7 minutes later caused their disappearance, but they returned 60 seconds later, then disappeared for a time, but returned from time to time for the next hour. During this period the acceleration of the heart action indicated that the peripheral vagus depressing effect of the atropine was still present.

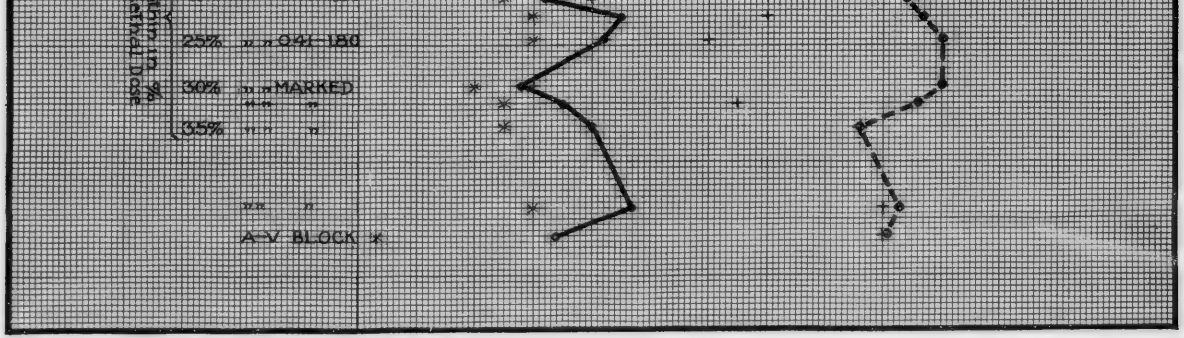
Whether it is correct to attribute the occurrence of these ectopic contractions to the previously administered strophanthin or digipuratum alone, or to a combination of the action of atropine and the other drug cannot be definitely determined. In favor of the latter explanation are the experiences in two experiments. In Experiment 83, although digipuratum had not been given for a considerable period (24 minutes and 3 minutes) each of the two injections of atropine was followed by ectopic beats, and in Experiment 64 atropine appeared twice to cause their occurrence. The explanation appears to be that the removal of vagus control permits the tertiary centers in the ventricle, already excited by



TEXT-FIG. 1. Variations in the sinus rate and P-R interval



e sinus rate and P-R interval under the influence of strophanthin and atropine.


DIGITALIS DOSE
25%
30%
35%
A-V BLOCK

strophanthin or digipuratum, to inaugurate ventricular contraction. Against this view must be considered the fact that in numerous other experiments under apparently similar conditions atropine did not cause the occurrence of ectopic contractions, but, as will be shown below, appeared at times to prevent them, or to cause their disappearance (Danielopolu (17)).[14]

In a number of experiments, when ectopic contractions appeared after the administration of strophanthin or digitalis, atropine caused them to disappear or to become less frequent (Fig. 4, D, E, and F). At times when large doses of strophanthin or digipuratum had caused a constant succession of ectopic beats, the administration of atropine was followed by a change in the shape of the ventricular complex so that it more closely resembled that of a nomotopic contraction (Fig. 5, D and E), but in other instances under apparently similar conditions this change did not occur. In Experiment 71, for instance, although at an earlier stage atropine caused the discontinuance of ectopic beats, it lost this power later. Both before and after atropine failed, this result was accomplished by giving amyl nitrite. In Experiment 73, after large doses of strophanthin had caused ectopic ventricular contractions, atropine produced about the same effects as did amyl nitrite. Early in the experiment both altered the complex somewhat but later produced little or no effect. A comparison of all the observations made with these two drugs demonstrates the fact that amyl nitrite possesses, to a much higher degree than does atropine, the power of inhibiting or altering ectopic ventricular contractions.

SUMMARY.

The Effects of Strophanthin and of Digitalis.

1. In intact dogs the dose which caused slowing and other signs of vagus stimulation without causing ectopic contractions usually

[14] Danielopolu (17) reports a case in which the administration of 1.5 mg. of atropine sulfate regularly relieved a bigeminy produced by digitalis or prevented its development even after doses of digitalis larger than those which regularly caused the appearance of bigeminy. In this instance the heart rate was increased markedly (from 70 to 130) by atropine. When the heart became slow again the bigeminy returned. In the experiments under discussion there was no such increase in the heart rate following the administration of atropine.

lay between 30 and 40 per cent of the minimum lethal dose for Thoms *g*-strophanthin[15] and for digipuratum[16] and a fluid extract of digitalis,[16] given intravenously in dilute solution in about 15 minutes.

2. In dogs strophanthin and digitalis when given intravenously produced typical alterations in the electrocardiogram almost immediately and with equal rapidity.

3. In this series the changes in the electrocardiogram following the administration of 25 to 40 per cent of the minimum lethal dose of strophanthin or digitalis often passed off within an hour or two and with few exceptions were not visible in electrocardiograms taken 20 hours later. The effects of digitalis did not appear to be more lasting than those of strophanthin. As there were marked differences in the persistence of the action of each of these drugs within its own series, this conclusion is provisional.

4. A persistent auriculoventricular block (without the occurrence of ectopic beats) was obtained only exceptionally, but the administration of 30 to 40 per cent of the minimum lethal dose of either of these drugs was usually followed by the occurrence either singly or together of occasional auriculoventricular blocks or exaggerated sinus arrhythmia or sinus block, and by pronounced slowing of the sinus rate.

5. Occasionally, with or without previous slowing or the occurrence of ectopic beats, these doses caused a marked acceleration of the sinus rate.

6. The auriculoventricular (P-R) time was usually lengthened but may be shortened.

7. The P wave may become less positive, isoelectric, or negative.

8. The T wave most often became negative before or during the stage of slowing but frequently became or remained positive during this stage. When the sinus rate increased or when occasional ectopic beats occurred, this wave almost always became decidedly more positive. At times it varied rapidly and decidedly within a period of a few seconds.

[15] 0.12 mg. per kilo.

[16] Twelve frog units per kilo.

9. Ectopic ventricular beats occurred in a number of instances after doses ranging from 19 to 40 per cent of the minimum lethal dose, and usually but not always when more than 40 per cent had been injected within 15 to 30 minutes.

10. Previous administration of either of these drugs did not appear to cause either a quantitative or qualitative alteration in the reaction of the heart to amyl nitrite or atropine (with the exception that such previous administration appeared responsible for the fact that in one experiment the inhalation of amyl nitrite, and in two others the injection of atropine, was followed by the occurrence of ectopic beats).

The Effects of Amyl Nitrite.

1. In intact dogs, both before and after the administration of strophanthin or of digitalis, the inhalation of amyl nitrite usually caused at first (probably largely reflexly) a pronounced increase of vagus tone shown by slowing of the sinus rate, exaggerated sinus arrhythmia, or auriculoventricular block. Later the sinus rate was accelerated and arrhythmia or blocks disappeared (probably due to vasodilatation and diminished vagus tone).

2. The auriculoventricular (P-R) time was usually lengthened at first and later was shortened.

3. The P wave often at first became shorter, isoelectric, or negative, but later became much more positive.

4. The T wave at first often became less positive or more negative, but later became strongly positive.

5. When the previous administration of not too large doses of strophanthin or digitalis caused the occurrence of ectopic ventricular beats, the inhalation of amyl nitrite usually caused their temporary disappearance. Even when large doses of these drugs caused a constant succession of ectopic contractions of various types, its inhalation was often followed by a temporary return of nomotopic cycles or by a change in the form of the ventricular complexes so that they more closely resembled that of nomotopic ones.

6. Only large amounts of amyl nitrite appear to have the power to prevent ectopic ventricular beats or to alter their type.

The Effects of Atropine.

1. In intact dogs before and after the administration of strophanthin or digitalis, small doses of atropine usually caused auriculoventricular block (or exaggerated it if already present). Larger doses, after first producing the same effect as the small ones, caused an increased sinus rate and abolished blocks or sinus arrhythmias if present.

2. The P-R time was usually lengthened by small doses and shortened by larger ones.

3. The P wave sometimes became shorter at first (less positive), isoelectric, or negative, but later (when the peripheral vagus paralysis ensued) it became positive again, often much more so than it was originally.

4. When ectopic contractions were present as a result of previous administration of strophanthin or digitalis, atropine, like amyl nitrite, often caused their disappearance, or altered the form of the ventricular complex, but was less efficient in this direction than amyl nitrite.

5. Occasionally the injection of atropine into dogs, which had previously received strophanthin or digitalis, appeared to facilitate or bring on the occurrence of ectopic ventricular contractions.

I wish to acknowledge my indebtedness to Dr. Alfred E. Cohn for his courtesy and assistance during the experiments and in the preparation of the manuscript.

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EXPLANATION OF PLATES.

In all the figures (except Fig. 9, A to G) divisions of the abscissæ equal 0.04 second. In Fig. 9, A to G the time is indicated by the lowest line, each interval representing 0.2 second. Divisions of the ordinates equal 10^{-4} millivolts. In Fig. 1, B to G the upper curve represents the blood pressure as recorded by a Harvard membrane manometer, a rise in the curve denoting a fall in the blood pressure. All the curves were made with Lead II (right fore leg to left hind leg).

PLATE 59.

Fig. 1, A to G (Experiment 85). In the upper curve, which is that of the blood pressure, a rise of the curve indicates a fall in the blood pressure.

A and B are controls. In A there is a spontaneous auriculoventricular block.

C was taken when amyl nitrite had been inhaled for 90 seconds. The blood pressure has fallen and as a consequence the heart beats more rapidly than in the control until this increase in heart rate leads to a rise in blood pressure which is followed by an auriculoventricular block. The P waves have become much taller than in the control curves.

D was taken 60 seconds after C when the inhalation had lasted 2½ minutes. The blood pressure has fallen in spite of the increased heart rate, and blocks and sinus arrhythmia have been abolished.

E was taken 1 minute after 29 per cent of the minimum lethal dose of a fluid extract of digitalis had been injected in 72 minutes. It shows an auriculoventricular block and a sinus arrhythmia.

F was taken 4 minutes after E when amyl nitrite had been administered for 3¼ minutes. The sinus rate has increased and the blood pressure has fallen decidedly, but the curve shows one auriculoventricular block and some sinus arrhythmia.

G was taken 30 seconds after F when the inhalation had lasted 3¾ minutes. The blood pressure has fallen still further, and arrhythmia and blocks are abolished. The sinus rate is markedly accelerated, the P–R interval is shorter, the P waves are much taller, and the T waves are more positive than in E.

PLATE 60.

FIG. 2, A and B (Experiment 56).

A is the control.

B shows auriculoventricular block with ectopic ventricular beats developing 6 minutes after the injection of 40 per cent of the minimum lethal dose of strophanthin. It also shows a negative T wave and the P-R interval increased to 0.17 second.

FIG. 3, A to E (Experiment 64).

A is the control. B and C serve also as controls to D and E.

B was taken when 71 per cent of the minimum lethal dose of strophanthin had been administered in 4 hours and 29 minutes. It shows slowing of the sinus rate and marked sinus arrhythmia with negative T waves.

C was taken 4 minutes and 20 seconds after B, and 80 seconds after the injection of 0.19 mg. of atropine sulfate. It shows abolition of sinus arrhythmia and marked acceleration of sinus rate, increase in height of the P waves, and change in form of the T waves. The P-R interval is almost unaltered.

D, taken 2 minutes and 50 seconds after C, shows ectopic ventricular beats.

E, taken 3 minutes after D, shows ectopic ventricular beats.

PLATE 61.

FIG. 4, A to F (Experiment 71).

A is the control.

B was taken after 44 per cent of the minimum lethal dose of strophanthin had been injected in 23 minutes. It shows ectopic ventricular beats.

C was taken 80 seconds after B. Amyl nitrite has been inhaled for 40 seconds. It shows the disappearance of ectopic beats.

D was taken 8 minutes after C. It shows a return of the rhythm seen in B. No additional strophanthin has been given.

E was taken 16 minutes after D; 5½ minutes after 50 per cent strophanthin had been injected in 43 minutes; 15 minutes after a first injection of atropine sulfate, 0.0125 per kilo; and 2 minutes after a second injection of the same size. It shows the altered form of the ventricular complex.

F was taken 3 minutes after E and 30 seconds after the injection of a third dose of atropine sulfate, 0.025 mg. per kilo (0.05 mg. per kilo in all). It shows normal cycles.

FIG. 5, A to E (Experiment 73).

A is the control.

B was taken 6 minutes after the injection of 96 per cent of the minimum lethal dose of strophanthin in 1 hour and 47 minutes. It shows ectopic ventricular beats of different types.

C was taken 90 seconds after B, when amyl nitrite had been inhaled for 60 seconds. It shows alteration in the form of the ventricular complexes.

D was taken 90 seconds after C. No amyl nitrite was given for 90 seconds. It shows the return to curves similar to those in B before inhalation of amyl nitrite.

E was taken 3 minutes after D, 30 seconds after the injection of atropine sulfate, 0.07 mg. per kilo. It shows ventricular complexes like those seen in C, following the inhalation of amyl nitrite.

PLATE 62.

FIG. 6, A to G (Experiment 83).

A is the control.

B was taken 4 minutes after 37 per cent of the minimum lethal dose of digipuratum had been injected in 2 hours and 25 minutes. It shows slowing of the rate with sinus arrhythmia.

C was taken 2 minutes after B. Amyl nitrite had been inhaled for 60 seconds. The curve shows abolition of sinus arrhythmia, acceleration of sinus rate, increase in height of P waves, and strongly positive T waves.

D was taken 19 minutes after C, 15 minutes after 39 per cent of the minimum lethal dose of digipuratum had been injected during 2 hours and 46 minutes.

E was taken 90 seconds after D and 30 seconds after the injection of atropine sulfate, 0.012 mg. per kilo. It shows slight variation in the height of the P waves and a marked change in the T waves which are much more positive than in D.

F was taken 2 minutes after E, 60 seconds after a second dose of atropine sulfate, 0.012 mg. per kilo. It shows three auriculoventricular blocks and further change in the T waves.

G was taken 5½ minutes after F. It shows ectopic ventricular contractions, alternating with cycles like those in F.

PLATE 63.

FIG. 7, A to D (Experiment 83).

A is the control.

B was taken 10 minutes after the injection of 20 per cent of the minimum lethal dose of digipuratum in 34 minutes. It shows ectopic beat and an auriculoventricular block, also tall T waves. The P-R interval is increased.

C was taken 4 minutes after B when amyl nitrite had been inhaled for 20 seconds. It shows an ectopic beat and an auriculoventricular block. The T wave has become less positive.

D was taken 20 seconds after C when amyl nitrite had been inhaled for 40 seconds. The sinus rate accelerated, the P-R interval shortened, and the T wave became strongly positive. The P waves are now all very tall.

FIG. 8, A, B, and C (Experiment 53).

A is the control.

B was taken 70 seconds after the commencement of inhalation of amyl nitrite. It shows an R-R interval of 4.02 seconds.

C was taken at the end of the inhalation, lasting 7 minutes and 40 seconds. It shows a greatly increased rate and regular rhythm. The P-R interval shortened to 0.08 second.

PLATE 64.

FIG. 9, A to G (Experiment 62).

A is the control.

B was taken immediately after the injection of 19 per cent of the minimum lethal dose of strophanthin. It shows an auriculoventricular block, an ectopic ventricular beat, a change to a negative T wave, and a slightly lengthened P-R interval.

C was taken 2 hours and 10 minutes after B. 36 per cent of the minimum lethal dose had been injected in 3 hours and 15 minutes. It shows exaggerated sinus arrhythmia, P waves varying from strongly positive to isoelectric, and negative T waves.

D was taken 4 minutes later; amyl nitrite had been inhaled for 30 seconds. The arrhythmia is abolished, the rate markedly increased, the P-R time shortened to 0.08 second, all P waves are strongly positive, and T waves changed from negative to diphasic.

E was taken 23 minutes and 20 seconds after D. 40 per cent of the minimum lethal dose of strophanthin has been injected in 3 hours and 42 minutes. It shows marked sinus arrhythmia, one blocked P wave, P waves varying from strongly positive to isoelectric, and T waves of variable degrees of inversion.

F was taken 3½ minutes after E and 2 minutes after the injection of 0.016 mg. of atropine sulfate per kilo. It shows much more frequent auriculoventricular blocks, P waves of varying heights, and a lessening in the negativity of the T waves.

G was taken 12½ minutes after F and 5 minutes after the second injection of 0.016 mg. of atropine sulfate per kilo. It shows marked acceleration of sinus rate, abolition of block, and almost complete merging of T and P waves, both of which are strongly positive.

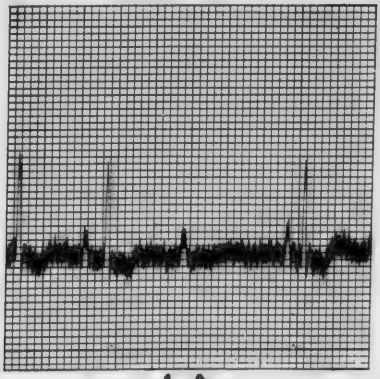
PLATE 65.

FIG. 10, A, B, and C (Experiment 68).

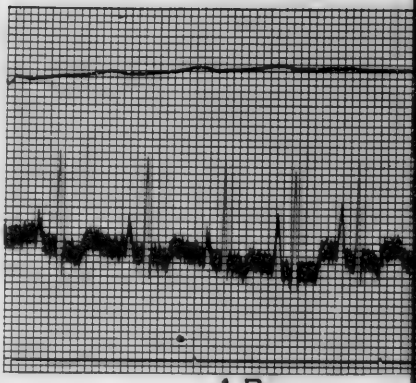
A is the control.

B was taken 30 seconds after the injection of atropine sulfate, 0.006 mg. per kilo. It shows an auriculoventricular block with negative P waves. It also shows P waves changing from negative to positive and back again to negative.

C was taken 7 minutes after B, and 35 seconds after the second injection of atropine sulfate, 0.006 mg. per kilo. It shows accelerated sinus rate, abolition of block and arrhythmia, shortened P-R interval, the P waves strongly positive, and change of the T wave to strongly positive.



1 A



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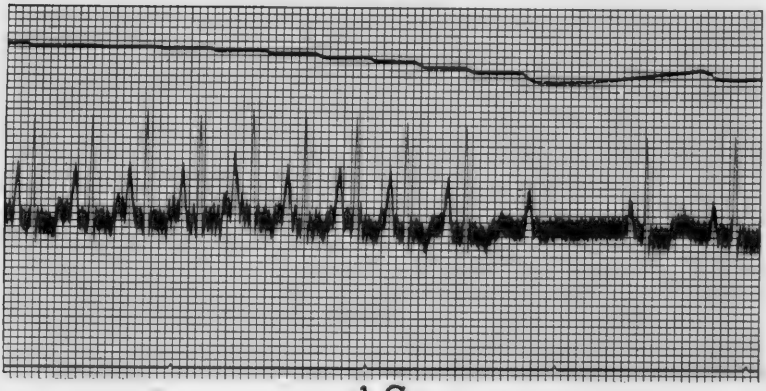

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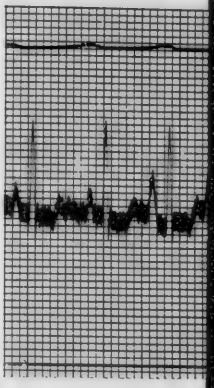
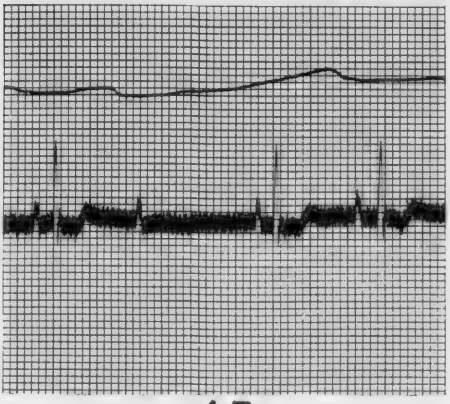
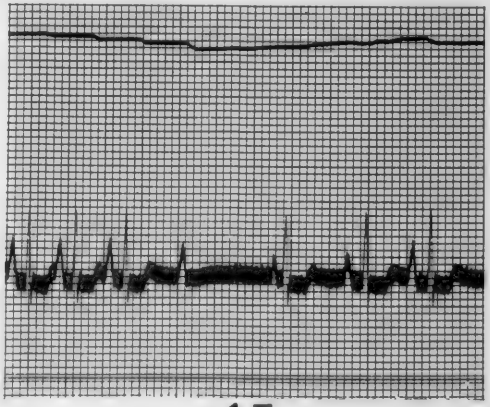




1 E



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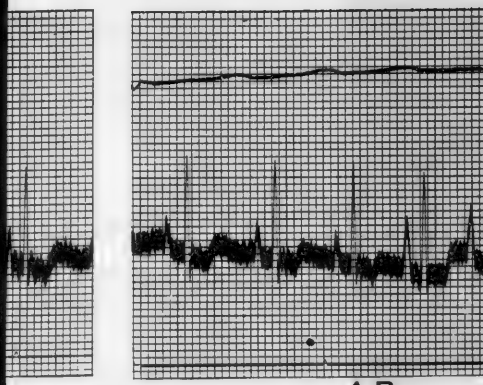

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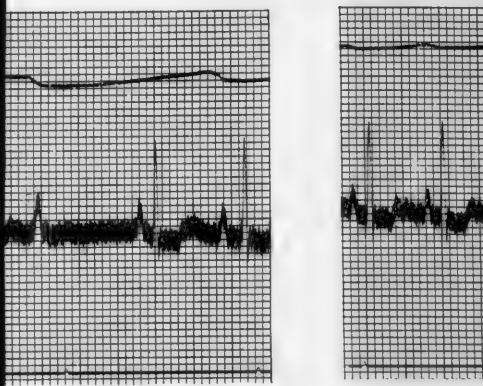
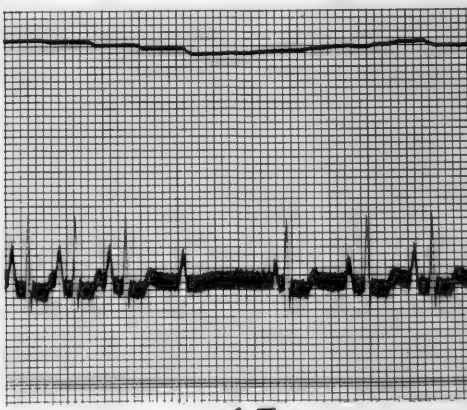
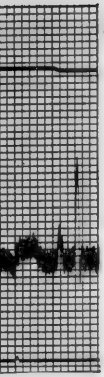
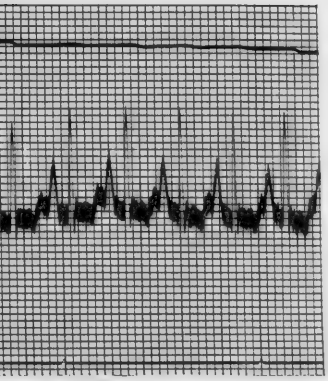




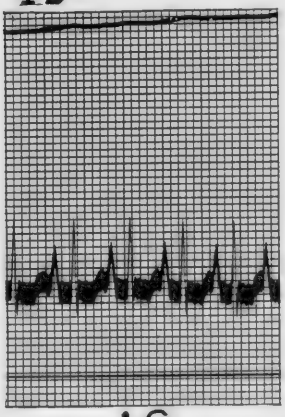
1F

PLATE 69.



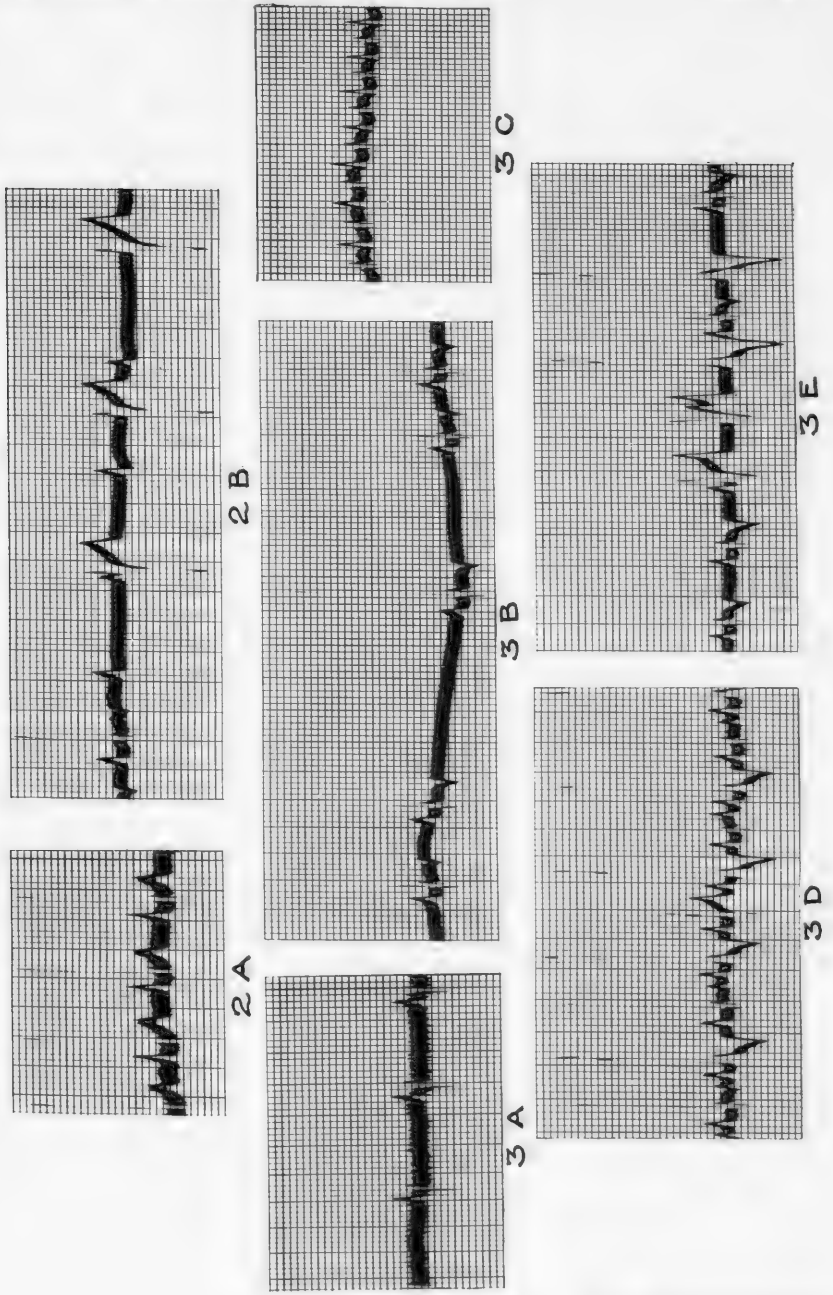


1 D



1 G

(Halsey: Digitalized Dog's Heart.)



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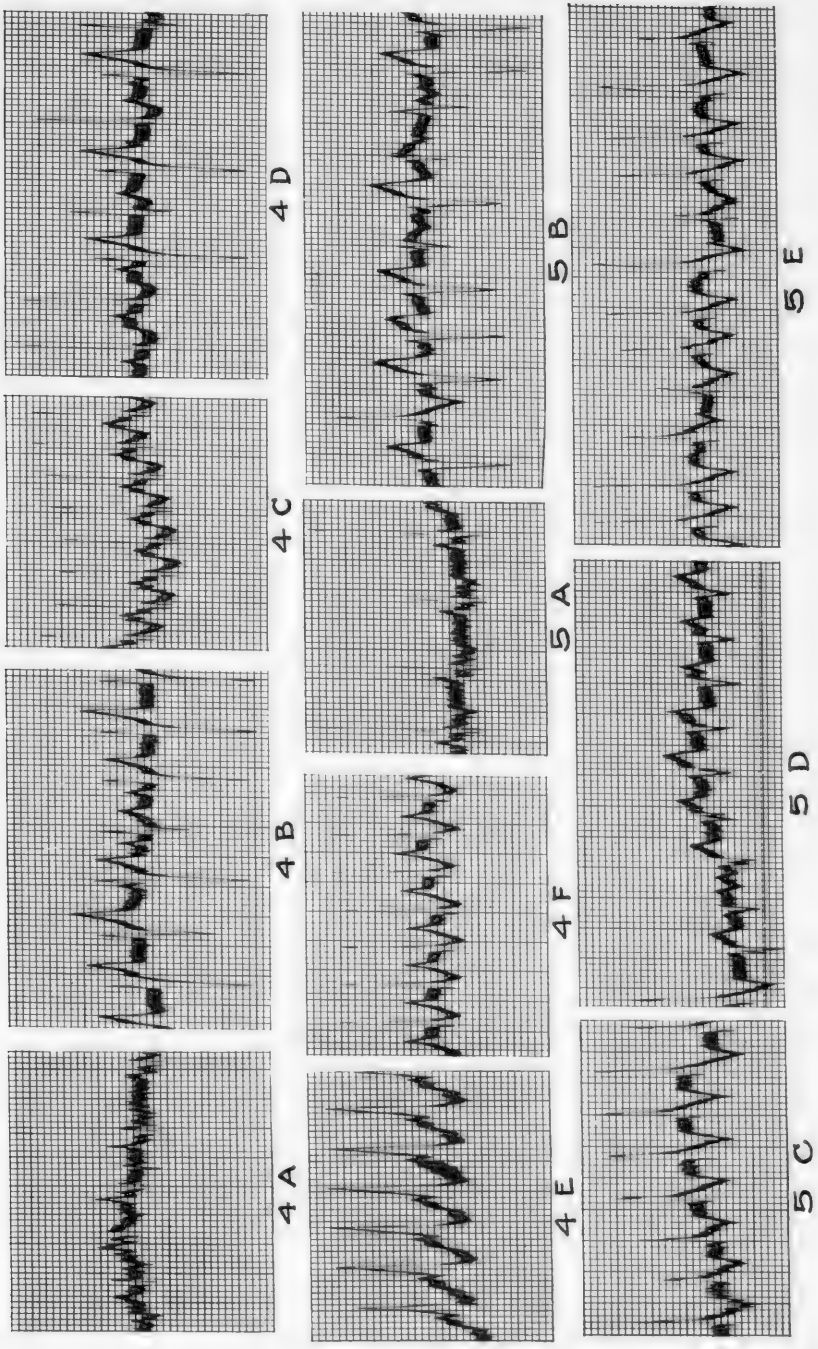

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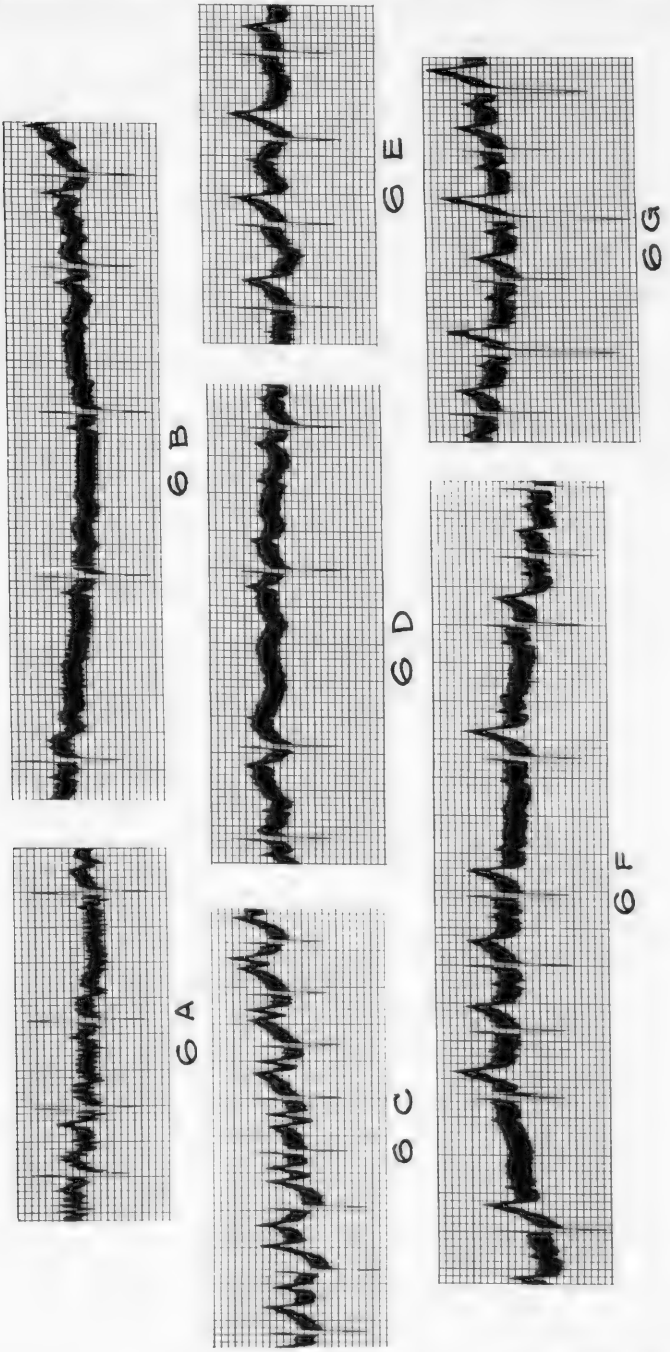
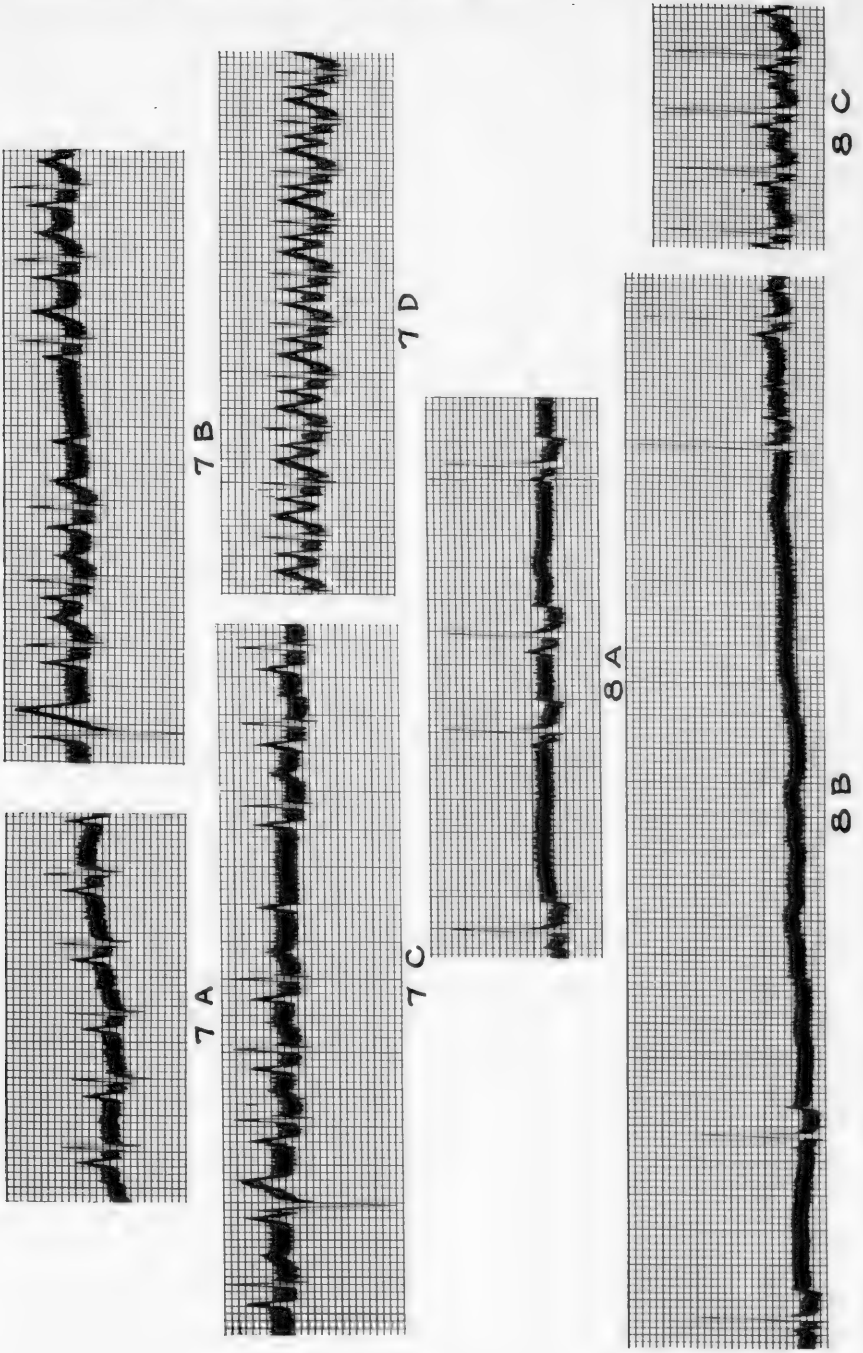
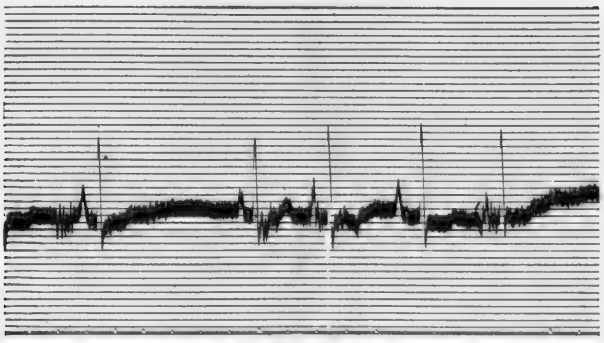


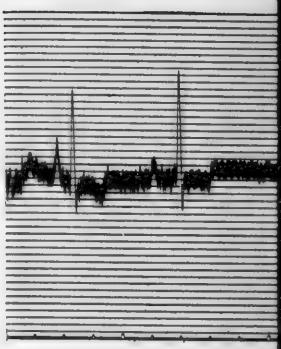
(Halsey: Digitalized Dog's Heart.)

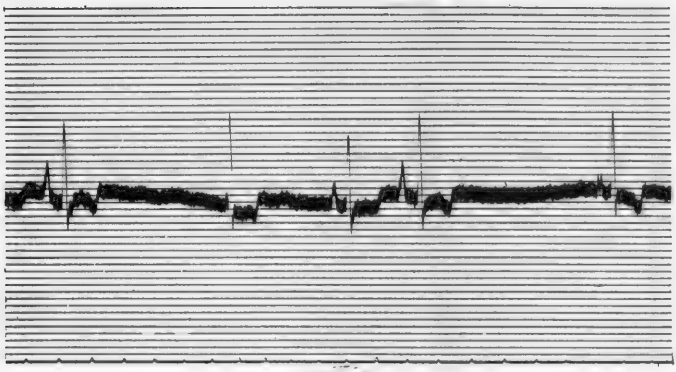


(Halsey: Digitalized Dog's Heart.)



9 A





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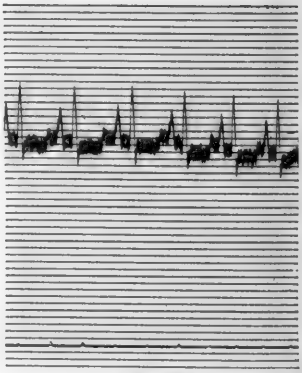

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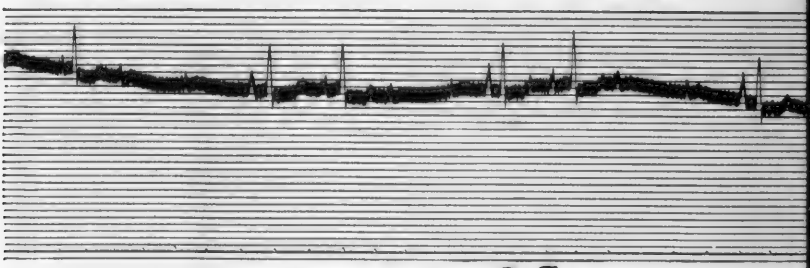
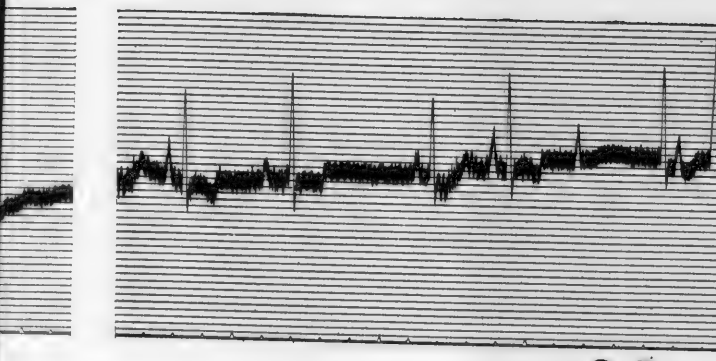
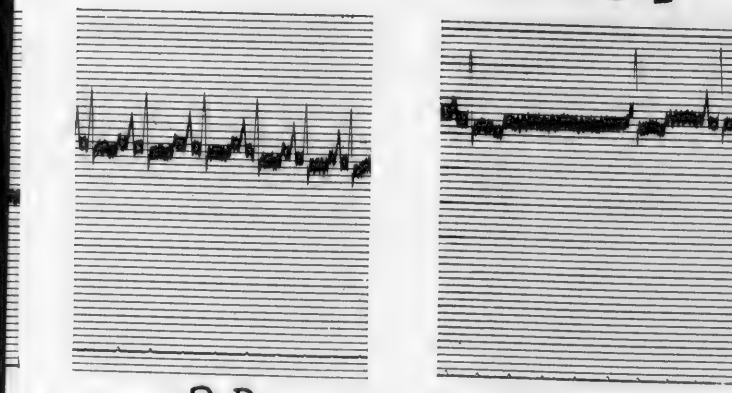


9 F



9 B



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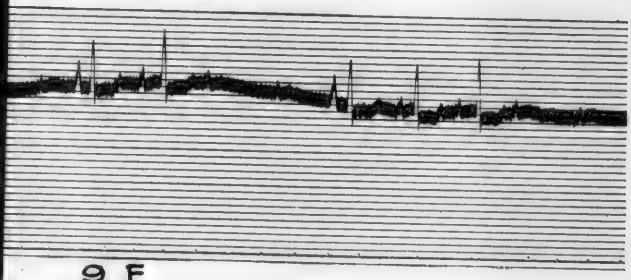
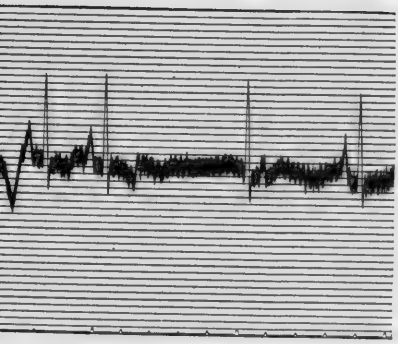
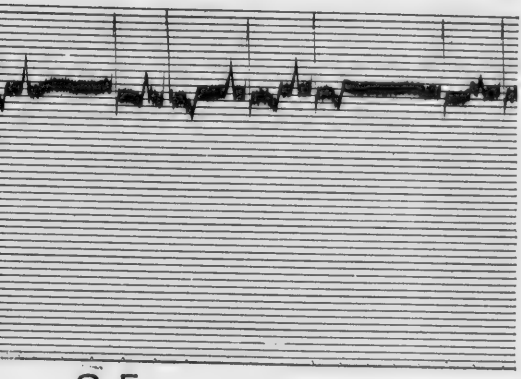


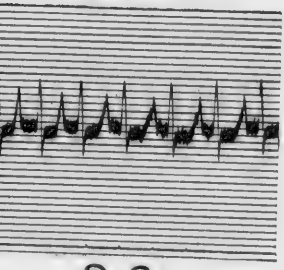
9 F

PLATE 64.



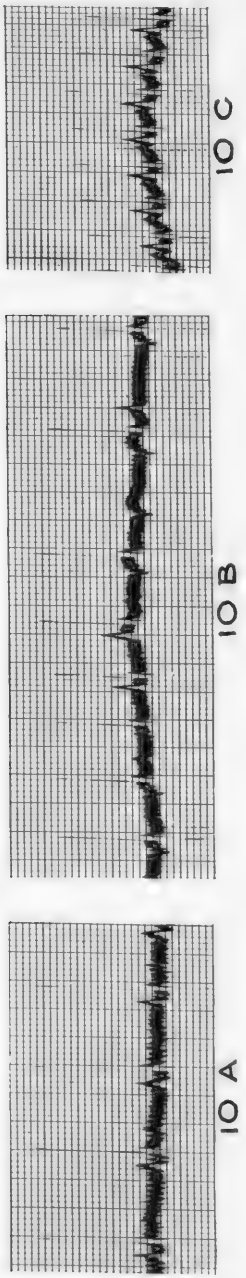


9 E



9 G

(Halsey: Digitalized Dog's Heart.)



(Halsey: Digitalized Dog's Heart.)

SPIROCHÆTA ICTEROHÆMORRHAGIÆ IN AMERICAN WILD RATS AND ITS RELATION TO THE JAPANESE AND EUROPEAN STRAINS.

First Paper.

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Infectious jaundice has been known for a long time to occur among troops in barracks, among sewer workers, miners, and rice planters, and its entity has been recognized (Larrey, Ozanam, 1849, Monneret, 1859, Laverau, 1865, Lancereau, 1882, Landouzy, 1882, Mathieu, 1886, and Weil, 1886). Weil described four cases with typical symptoms, and the disease is very often called by his name. Weil's disease is characterized by sudden onset of malaise, often intense muscular pains, high fever for several days, followed by jaundice and the appearance of bile pigments, albumin, and casts in the urine; in severe cases epistaxis, subcutaneous hemorrhages, and lymphadenitis are observed.

A disease supposed to be identical with the Weil's disease of Europe is prevalent in Japan, and in 1914 Inada and Ido succeeded in transmitting to guinea pigs the typical experimental disease, accompanied by jaundice, hemorrhages, albuminuria, etc., by inoculating them with the blood of patients suffering from the Japanese form of infectious jaundice. In the blood and various organs of the animals they discovered a new spirochete, which they also found in the human specimens. *Spirochæta icterohæmorrhagiæ*, as it was designated by Inada and his associates,[1] has since been proved beyond doubt to be the causative agent of the disease in question. A year and a half later, Uhlenhuth and Fromme,[2] and also Hübener and Reiter,[3] showed that the Weil's disease prevalent among the German soldiers during the present war could also be transmitted to guinea pigs by injecting them with the blood of patients. It was not until 1916, however, that Hübener and Reiter[4] announced the finding of a spirochete in the experimental animal material (liver, blood, kidney, etc.), which they called *Spirochæta*

[1] Inada, R., Ido, Y., Hoki, R., Kaneko, R., and Ito, H., *J. Exp. Med.*, 1916, xxiii, 377.

[2] Uhlenhuth and Fromme, *Med. Klin.*, 1915, xi, 1202, 1264, 1296, 1375.

[3] Hübener and Reiter, *Deutsch. med. Woch.*, 1915, xli, 1275.

[4] Hübener and Reiter, *Deutsch. med. Woch.*, 1916, xlii, 1.

nodosa. Following the publication in America[1] of the article by Inada and his collaborators, Stokes and Ryle[5] succeeded in transmitting the disease to guinea pigs by inoculating them with the blood of British soldiers in Flanders who had Weil's disease, confirming at the same time the presence of a spirochete closely resembling the *Spirochæta icterohæmorrhagiæ* of the Japanese workers. Stokes, Ryle, and Tytler[6] left the question of the identity of the two strains (the Belgian and the Japanese) open for future discussion. In the meanwhile, Martin and Pettit[7, 8] Costa and Troisier,[9, 10] Garnier and Reilly,[11] Renaux,[12] Merklen and Lioust,[13] Ameuille,[14] Salomon and Neveu,[15] and others, working among the French soldiers on the western front, reported similar clinical and experimental findings. They also considered the spirochete isolated from the French specimens to be closely related to the Japanese strain. On the Italian front numerous cases of jaundice have been observed, and Monti[16] has demonstrated the spirochete in the experimental material. According to MacKenzie, there were at one time a considerable number of cases of infectious jaundice among the Canadian soldiers stationed at Salonica.[17] It is of interest to note that while the mortality among the Japanese is as high as 38 per cent, that of the European soldiers did not exceed 2 to 3 per cent. It seems reasonable to assume that the Japanese strain has already acquired a marked increase in virulence for human subjects, owing probably to a more frequent passage from man to man. Such transmission is more frequent in Japan, for example, among the rice planters and miners with imperfectly protected feet, than in Europe, where exposure to the infection was brought about only through the unsanitary conditions of war.

The entrance of this spirochete into the human body seems to be of comparatively recent occurrence. The discovery of the spirochete in apparently healthy wild rats caught near the infected districts

[5] Stokes, A., and Ryle, J. A., *J. Roy. Army Med. Corps*, 1916, xxvii, 286.

[6] Stokes, A., Ryle, J. A., and Tytler, W. H., *Lancet*, 1917, i, 142.

[7] Martin, L., and Pettit, A., *Presse méd.*, 1916, 569.

[8] Martin and Pettit, *Bull. Acad. méd.*, 1916, lxxvi, 247.

[9] Costa, S., and Troisier, J., *Compt. rend. Soc. biol.*, 1916, lxxix, 1038.

[10] Costa and Troisier, *Bull. et mém. Hôp. Paris*, 1916–17, xl, series 3, 1928.

[11] Garnier, M., and Reilly, J., *Bull. et mém. Hôp. Paris*, 1916–17, xl, series 3, 2249.

[12] Renaux, E., *Compt. rend. Soc. biol.*, 1916, lxxix, 947.

[13] Merklen, P., and Lioust, C., *Bull. et mém. Hôp. Paris*, 1916–17, xl, series 3, 1865.

[14] Ameuille, P., *Bull. et mém. Hôp. Paris*, 1916–17, xl, series 3, 2281.

[15] Salomon, M., and Neveu, R., *Compt. rend. Soc. biol.*, 1917, lxxx, 272.

[16] Monti, A., *Boll. Soc. med. chir. Pavia*, 1916, Nos. 3–4.

[17] Personal communication.

seems to support the theory that the disease was originally epizootic among certain rodents, particularly wild rats, and after a long sojourn in this species of hosts its virulence for these animals has been reduced to such an extent as to cause the latter no inconvenience, or, at least, a state of tolerance for the spirochete has developed.

Inada, Ido, Hoki, Ito, and Wani[18] found nearly 30 per cent of all the rats examined to be infected; that is, to carry the spirochete in their kidneys. Stokes Ryle, and Tytler,[6] as well as Martin and Pettit,[19] were able to demonstrate the spirochete in rats captured on the western front, not only in those taken in the immediate neighborhood of the infected zone, but also in rats captured in localities some distance from the trenches.

In America, especially the United States, there have been few epidemic or endemic cases of infectious jaundice reported from various quarters of the continent (Toronto, Middle Western and Southern United States) and from Cuba, but it was not known whether or not these cases corresponded with those found in Europe and Asia. The discovery of the specific pathogenic agent now enables us to answer this question experimentally.

Isolation of the Organism.

We have collected a large number of wild rats in this country and removed their kidneys for the purpose of ascertaining whether or not the organs contained the spirochete which causes the typical experimental lesions characteristic of the organism of infectious jaundice. Leaving the experimental details for a future communication, it may be well to state here briefly that by inoculating the emulsion made of the kidneys of forty-one wild rats into fifty-eight guinea pigs during the last 3 months, we have been able to produce in three groups of guinea pigs (four in each group) a typical icterohemorrhagic spirochetosis altogether identical with the findings in the guinea pigs which died of the injections of the Japanese and Belgian strains of *Spirochæta icterohæmorrhagiæ.*

Since it was our practice to make an emulsion from the kidneys (eight) of four wild rats and inject the same into four to six normal

[18] Inada, R., Ido, Y., Hoki, R., Ito, H., and Wani, H., *J. Exp. Med.*, 1916, xxiv, 485.

[19] Martin and Pettit, *Compt. rend. Soc. biol.*, 1917, lxxx, 10.

guinea pigs, it is difficult to say whether the icterohemorrhagic spirochetosis produced in some of the guinea pigs in each group was due to one or more of the rats employed. It would have been better if we had inoculated several guinea pigs with the emulsion of kidneys from each rat, but this would have involved a large number of guinea pigs, and hence our method of mixing four in each group. Since the successful isolation of the organisms from three of the pooled groups, we have resorted to individual tests, the results of which will be reported later.

The strain of spirochete isolated from the American wild rats caught in the vicinity of New York City produced death in guinea pigs within 9 to 12 days, attended by marked jaundice, cholemia, choluria, and extensive hemorrhages in various viscera. The temperature seldom exceeds 39°C.; that is, it does not show the fever which usually precedes the collapse and the appearance of jaundice in the guinea pigs inoculated with the Japanese or Belgian strains, which have at this time been passed through many guinea pigs. The spirochetes are found to be abundant in the internal organs, but are only moderately numerous in the blood. In the urine of some guinea pigs, which succumbed to the experimental icterohemorrhagic spirochetosis, varying numbers of the organisms were found in the urinary casts.

The morphology of the organism corresponds with that of the Japanese and Belgian strains, with which we were able to compare it.[20] Its elementary structure is that of a closely wound slender cylindrical thread with gradually tapering ends, averaging 9 by 0.25 μ. Individuals of 3 to 4 μ or 20, 30, and even 40 μ are met with in a culture. The number of coils is greater in a given length than that of any spirochete hitherto known. It is so closely wound that within 5 μ there are 10 to 12 coils. Near the extremities, the coils become closer. They are never very deep, and in general, the aspect of the whole body is that of a transversely barred chain of streptococci. The winding is rarely seen distinctly, although it can be brought out well by a carefully fixed stained preparation (osmic vapor fixation

[20] I am greatly indebted to Dr. Victor G. Heiser for the Japanese strain and to Dr. Alexis Carrel for the Belgian strain. The Japanese strain was kindly furnished by Dr. M. Miyajima, and the Belgian by Dr. Carl Browning, who obtained it from Dr. Adrian Stokes.

and Giemsa stain), or under powerful dark-field illumination. It should be noted that the description of the organism by most authors leaves this point unclear, and so far no satisfactory microphotograph has been reproduced. The movement and customary position of the organism in a free space are characteristic. Active specimens show a straight body with one or both ends curved in the form of a semicircle. The length of the hook at the end varies somewhat but is usually about 3 to 5 μ. While in motion, the organism, without relaxing its elementary minute windings, rotates around its axis, making about two to four turns per second, giving the impression of a drawn out figure eight. The movement is bipolar, and its direction alternates at short intervals. When passing through a semisolid medium, such as fibrin or soft agar, the body of the spirochete assumes a wavy spiral not unlike the *refringens* type. The number of waves may vary from a few broad ones to as many as five or six in some of the long individuals found in a culture. The movements are brusque and erratic, changing suddenly the direction of progression. At times, the organism moves about with one end taking various directions. The facility with which these spirochetes travel through the fibrin or semisolid agar medium is without a parallel among any known spirochetes. The body is absolutely flexible. There is a distinct halo around the organism, but no membrane has so far been demonstrated. The part of the body which forms the hook terminates in a fine point, but no minute flagellum-like projection could be demonstrated by staining (Loeffler, Pitfield, Casares-Gil, Fontana, etc.), or by dark-field illumination. It is devoid of a terminal filament such as is characteristic of a spironema or treponema, and is resistant to saponin (10 per cent), unlike all other spirochetes. It calls for a new genus, and on account of its fine and minute windings, the name *Leptospira* is suggested.

The hooked ends form one of the most characteristic poses of the organism while rotating on its axis in a free space, but as soon as it meets a solid or semisolid obstacle, it begins to penetrate into it. Its habitat seems to be a porous gelatinous mass of substance, the organisms swarming in and out of it. In a culture the majority of the organisms will be found in a semisolid piece of medium. After death it may retain its position at the moment of death; hooked or

contorted forms, resembling the letters c, s, l, and b, are most frequently met with among immobile or dead organisms. When destroyed by a strong acid or alkali, they lose their minute curves, swell up, and become indistinct and straight. The organism has the power of almost perpetual motion. A culture as old as 3 months still displays as much vigor as does a young one. None of the other varieties of spirochetes have this property in cultures. The organism passes the Berkefeld candle V.

Cultures.

The Japanese investigators[1] succeeded in obtaining a culture of their strains by using a medium similar to that previously introduced by the writer for the spirochete of relapsing fever.[21] Ito and Matsuzaki approved this technique and recommended also the use of blood gelatin or blood agar in various concentrations.[22] In Europe, Reiter[23] cultivated the German strain in the sera of various animals, sometimes diluted several times with isotonic salt solution. Recently, Martin, Pettit, and Vaudremer[24] obtained a culture of the French strain by the use of animal sera, particularly that of beef, diluted with Locke's solution. In all these instances, the surface of the culture medium was covered with a layer of sterile paraffin oil, as recommended by the writer for the cultivation of spirochetes in general. The Belgian strain of Stokes has not yet been cultivated, and Stokes, Ryle, and Tytler particularly laid stress on its resistance to artificial cultivation as a characteristic of the strain.

The writer has been interested in determining the cultural conditions which will give uniform results, and has found that the following methods give the most successful growth, not only with the Japanese, but also with the Belgian (Stokes) and American strains, which were obtained in permanent cultures for the first time by these means.

In our experience, it seemed best to make a distinction between the initial culture or the first generation and the subcultures, since there is a great difference in the readiness with which the first and subsequent generations of the spirochetes grow in culture. For example, all three strains, in our hands, failed to grow, or grew poorly

[21] Noguchi, H., *J. Exp. Med.*, 1912, xvi, 199.

[22] Ito, T., and Matsuzaki, H., *J. Exp. Med.*, 1916, xxiii, 557.

[23] Reiter, *Deutsch. med. Woch.*, 1916, xlii, 1282.

[24] Martin, L., Pettit, A., and Vaudremer, A., *Compt. rend. Soc. biol.*, 1917, lxxx, 197.

in the various media recommended by different authors. The best procedure, and one which has always been reliable for securing initial growth was to produce strands of loose fibrin in the fluid culture media by using a small quantity of citrate plasma in combination with the diluted or undiluted serum of a suitable animal. The beneficial effect of a loose fibrin upon the culture of the spirochetes of relapsing fever has already been mentioned by the writer.[21] The dilution of the serum may be made in any proportion above 1:10 by adding a sterile saline (0.9 per cent) Ringer solution, or even plain water. For obtaining the spirochetal material for inoculation, the citrate blood derived from the heart of a guinea pig having the disease is best, although an emulsion of the liver or kidney may also be used. When no secondary bacteria are present, a positive culture can be secured at the first attempt. By using graduated quantities of the infected guinea pig's blood (citrate), the writer secured a good growth in as high a dilution as 1:100,000.

Undoubtedly blood cultures for diagnostic purposes in human cases are feasible with a suitable medium. For this purpose I should recommend two different media, one being apparently as good as the other: (*a*) rabbit serum 1 part + Ringer or 0.9 per cent sodium chloride solution 3 parts + citrate rabbit plasma 0.5 part, covered with a thin layer of sterile paraffin oil; (*b*) the same, except for the use of 0.5 to 1.0 part of neutral or slightly alkaline agar (2 per cent), which should be added while in a liquid state and quite hot (60–65°C.) in order to get a uniform mixture of the agar. These culture media, because of the paraffin oil layer, can be preserved at room temperature for many months, though a cooler place is better. They may be inoculated with suspected blood by introducing a quantity which is regarded as adequate in each instance. In the case of an infected guinea pig, the detection of the spirochete in the blood can be made within 48 to 72 hours, if the culture tubes are placed at 30–37°C. The search for the organism should be made within the aerobic zone immediately below the surface (1.0 to 1.5 cm.), because, according to the experience of the writer, the organism is an obligatory aerobe, unable to grow in the absence of oxygen. This would explain the unsuitability of a solid medium, which prevents access of air.

The use of fresh tissue, as in the case of the relapsing fever spiro-

chete, is not required for the cultivation of this organism, and is perhaps seriously detrimental in a fluid medium. Growth takes place at any temperature from 10° to 37°C., being much more rapid at higher than at lower temperatures. The growth is almost invisible in a fluid medium, but appears as a somewhat distinct haze in semi-solid medium such as described above (*a* and *b*). The appearance is not unlike the hazy, diffuse growth of various treponemata in similar media, the only difference being that the haze in the case of the latter stops abruptly a few cc. below the surface, while the present organism is unable to grow beyond a few cc. below the surface. So far, no investigator has mentioned the visible growth of the spirochete, all describing it as invisible.

Virulence.

The American strains possess an average degree of virulence, having killed guinea pigs in 9 to 12 days in the first generation in this animal. The lesions, as well as the jaundice, were severe and typical in every respect. The second passage killed the guinea pigs in a much shorter period, 6 to 8 days, the lesions being similar to those of the first passage. Again there was no fever. They seem to be running a course similar to that of the Japanese strain in attaining a higher virulence by successive passages. The virulence of the Belgian strain appears to be less readily increased, as the guinea pigs survive 9 to 10 days. This distinction in virulence may be due to the fact that the Belgian strain was brought to us in infected rats, instead of in guinea pigs, in which the Japanese strain was received.

Immunity Relations.

Reserving the experimental details for a fuller report, it may be stated briefly that the guinea pigs which had been made immune to the Japanese or Belgian strain resisted the inoculation of the liver emulsion containing large numbers of the spirochetes of American origin, while control animals succumbed to the typical infection in 6 to 8 days. The blood sera from guinea pigs and rabbits immunized with the Japanese or Belgian strain agglutinated the American strain as strongly as they did their own strains. The immune sera exerted

germicidal or lytic action upon the three strains indiscriminately. Whether or not protection experiments in guinea pigs will show any distinctions between the Belgian and the Japanese strains on the one hand and the American on the other will shortly be determined. As far as the former are concerned, the active immunity developed in guinea pigs or rabbits and their immune sera are effective, the one against the other, in almost equal titers.

The finding of the causative organism of infectious jaundice among wild rats in America, and the identification of this strain with those found in Asia and Europe seem to be particularly important in revealing a latent danger to which we have been constantly exposed but from which we escape as long as sanitary conditions are not disturbed by untoward events.

SUMMARY.

The principal points brought out in the present article are the following.

1. Wild rats captured in this country carry in their kidneys a spirochete which possesses the morphological and pathogenic properties characteristic of *Spirochæta icterohæmorrhagiæ* discovered by Inada in the Japanese form of infectious jaundice.

2. Cultures of the American, Belgian, and Japanese strains of the spirochete were obtained by a special technique described, the first two strains having been cultivated artificially for the first time.

3. Animals actively immunized against the Japanese strain resist inoculation, not only of the same strain, but also of the Belgian and American strains. The Belgian strain produces immunity equally effective against all three strains. Experiments to ascertain whether the immunity afforded by the American strain also protects against the Japanese and Belgian strains are in progress.

4. These findings warrant the conclusion that the spirochetes designated here as the Japanese, Belgian, and American strains are probably identical.

5. On account of its distinctive features, a new genus, *Leptospira*, has been suggested as the designation of this organism.